EXHIBIT 2

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TITLE
CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS

U.S. DEPT. of COMM./Pat. & TM Office — PTO-436L (rev. 10-78)

PARTS OF APPLICATION FILED SEPARATELY

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PATENT APPLICATION SERIAL NO. 08/088439

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APPLICANT

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TITLE

CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS

This is to certify that annexed hereto is a true copy from the records of the United States Patent and Trademark Office of the application which is identified above.

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08/088439

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Date: July 7, 1993

In re Application of

Inventor(s) : Lenore Kelly, Dean S. Burgi, Robert John Nelson

Title : CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS

Assignee : Thermo Separation Products (California) Inc.
Docket No. : SPA 096 PA

Hon. Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

Enclosed are the following papers:

__X__ Specification, abstract and claims, __19__ pages total
__X__ Drawings __7__ sheets
__X__ Declaration and Power of Attorney
_____ A claim for priority under 35 USC §119 is hereby made with respect to _____ application No. _____ filed _____. A certified copy of that application _____ is enclosed/_____ will be filed later.

Calculation of Fee

Basic Fee				$710.00
Total Claims	19 - 20 = 0	X $22.00	=	00.00
Independent Claims	2 - 3 = 0	X $74.00	=	00.00
Charge for Multiple dependent claims ($230.00)			=	00.00
		TOTAL		$710.00

Enclosed is our check in the amount of $710.00 which represents the payment of the above filing fee(s).

Respectfully submitted,

KILLWORTH, GOTTMAN, HAGAN & SCHAEFF

By _____
Timothy W. Hagan
Reg. No. 29,001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of

Applicant(s): Lenore Kelly, Dean S. Burgi, Robert John Nelson

Title : CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS

Docket : SPA 096 PA

Hon. Commissioner of Patents
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"Express Mail" Mailing Label Number HB37261124X US
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07/088439

CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS

Background of the Invention

The present invention relates to the separation and detection of common anionic species using capillary electrophoresis, and more particularly to the use of temperature control in a capillary electrophoresis system to improve separation and reproducibility for the detection of such anionic species.

The separation and/or detection of ionic species in chemical analysis is generally carried out using the electrochemical properties of the analytes. These properties may include ionic interactions and conductivity in ion chromatography and ionic mobility in capillary electrophoresis. Capillary zone electrophoresis (CZE) is a powerful and efficient method to separate small analytes at very low concentration levels by exploiting the different mobilities of sample components in an electric field.

A conventional CZE system typically includes a buffer-filled capillary column with inlet and outlet ends disposed into two reservoirs. The buffer is generally an electrically conductive medium, sometimes termed the "carrier electrolyte". The system also includes means for introducing the sample into the capillary column, an on-column detector for sensing the sample zones as they pass the detector, and a high voltage source to apply a voltage to the capillary column to cause migration and separation of the sample into identifiable components. The ionic species in the sample move from one electrode toward the other in the capillary column at a rate which is dependent, inter alia, upon the electrical charge, molecular size, mobility of those ions, and/or field strength.

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However, many analytes, including most inorganic ions, do not absorb ultraviolet or visible light. As capillary electrophoresis systems generally use direct photometric techniques such as ultraviolet/visible light (UV-VIS) detectors, the ions pass by the detector without being observed. These ions can be detected, however, using the technique of indirect photometric detection. Indirect photometric detection relies on the presence of light-absorbing buffer electrolyte ions in the sample. It is the absorbance of these buffer electrolytes which is monitored by the detector, not the absorptivity that the sample components may display.

Because the solution in the capillary is constrained to remain electrically neutral, sample ions displace the light-absorbing buffer electrolyte ions on a charge-for-charge basis as the sample migrates through the capillary. As the buffer electrolyte ions are displaced by the sample ions, more photons pass through to the detector. This increase in light throughput is recorded by the detector as a decrease in absorbance and is characterized by a negative peak. The magnitude of the negative peak is dependent upon the concentration of the displacing ion, the ratio of the negative charges on the buffer electrolyte ion to the sample ion, and finally, the concentration and extinction coefficient of the buffer electrolyte. Thus, non-absorbing ion species in the sample can be detected, and their concentrations determined using this technique.

Methods using indirect photometric detection in capillary electrophoresis have been described in the published literature, for example, by Foret et al., J. Chromatography, 470:299-308 (1989), and Kuhr et al., Anal. Chem., 60:2642-2646 (1988) and 60:1832-1844 (1988). Jones et al., U.S. Patent Nos. 5,104,506, 5,128,005, and 5,156,724, also describe the use of indirect photometric techniques for

the separation and detection of samples containing mixtures of common ionic species. However, problems remain in attempting to resolve and identify certain ion mixtures because some ions have similar migration rates and transparency to or absorbance of ultraviolet light.

To improve sensitivities for detection of certain ions below concentrations of about 1 ppm, other techniques such as electrokinetic injection, the use of electroösmotic flow modifiers, and changes in pH have been used. However, the need still exists in the art for improved methods in detection techniques in capillary electrophoresis, both to improve the separation and detection of very small concentrations of ion species, especially those which are nonabsorbers of light.

Summary of the Invention

The present invention meets those needs by providing an improved method for the separation of anions using capillary electrophoresis techniques. Both organic and inorganic anions may be separated. Using precise control of the temperature of the fluid in the capillary column, the migration speed and order of migration of the anions may be controlled to improve the selectivity of the process. Because the viscosity of the electrolyte solution in which the sample ions migrate is influenced by temperature, close temperature control provides a high degree of reproducibility for samples and enables one to track and identify specific anions.

In accordance with one aspect of the present invention, a method for detecting and separating anions in a sample using capillary electrophoresis is provided and includes the steps of providing a capillary filled with a negatively-charged carrier electrolyte, heating or cooling the capillary

to a target temperature different from ambient temperature, introducing a sample containing one or more anions into the capillary, applying an electrical current to the capillary under conditions causing anions in the sample to migrate and separate, and detecting the anions while maintaining the temperature in the capillary to within ±0.5°C of the target temperature. Detection of the anions may be by direct or indirect techniques. For example, the anions may be detected by a conductivity detector or a mass spectrometer, or by indirect techniques using a UV/visible detector. In a preferred embodiment of the invention, the carrier electrolyte contains a light-absorbing co-anion, and the anions are detected indirectly using a photometric detector.

By "carrier electrolyte" or "buffer", we mean any electrically conductive fluid medium for the sample. By "light-absorbing co-anion", we mean salts of anionic species known in this art to absorb visible or ultraviolet light including, but not limited to, chromate, vanadate, phthalate, pyromellitate, benzoate, and singly or multiply-charged carboxylate salts.

The method of the present invention may also be carried out by including an electroosmotic flow modifier in the carrier electrolyte which controls the speed and/or direction of the electroosmotic flow of the carrier electrolyte. While there are many electroosmotic flow modifiers known in the art, for use in this invention, preferred electroosmotic flow modifiers include diethylenetriamine (DETA) and aliphatic trimethyl ammonium halides or hydroxides such as tetradecyltrimethylammonium bromide (TTAB).

The present invention is particularly suited to detect such common low molecular weight inorganic and organic anions such as chloride, nitrate, nitrite, sulfate, and oxalate. Many of these anions have been difficult to detect using conventional CZE techniques in the past because they do not

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absorb significant amounts of light at many wavelengths in the ultraviolet range. The present invention also provides for the detection of such other common organic anionic species as tartrate, malate, succinate, lactate, acetate, propionate, butyrate, citrate, and caprylate. The use of light-absorbing co-anions in a preferred embodiment which are displaced as the other anions in the sample migrate, permits their presence to be detected as an absence of UV light absorption or a negative peak monitored on the photodetector.

By proper selection of a target temperature which is different from ambient temperature and which may be higher or lower than ambient, and closely controlling the temperature of the fluid in the capillary column to within ±0.5°C, and most preferably to within ±0.1°C, these anionic species may be separated and detected at very low concentrations of less than 1 ppm, and preferably less than 100 ppb. A preferred target temperature is in the range of from about 25° to 60°C. At a selected temperature within that range, the speed and order of migration, and thus the selectivity of the separation of the anions may be precisely controlled. Further, by programming a temperature increase into the separation process, critical early (negative) peak resolution of fast migrating anions may be preserved while speeding the migration of later (negative) anionic peaks. Thus, both the speed and resolution of the method are enhanced. Additionally, precise temperature control provides a high degree of reproducibility for the process.

In another embodiment of the invention, anions in a sample may be detected by simultaneously monitoring the sample at two different wavelengths with the photodetector. This method takes advantage of the behavior of nitrogen-containing anions such as nitrates, nitrites, and thiocyanates. In this embodiment, the sample is monitored at wavelengths of 210 and 254 nm simultaneously. Nitrate and

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nitrite strongly absorb at 210 nm, but not at 254 nm. Thus, a strong positive peak occurs at the lower wavelength, while a negative peak is simultaneously observed at the higher wavelength. These unique signatures permit ready identification of nitrogen-containing anions.

In yet another embodiment of the invention, different portions of the same sample are run sequentially, and the temperature in the column for each sample portion is changed. In this manner, the order of elution of the anions in the later sample portions may be compared with the order in the first portion. For example, at a capillary temperature of 20°C, nitrate will elute from the capillary column prior to sulfate. At capillary temperatures of 40°C and above, that order of elution is reversed and can be observed so that these ions may be readily identified and tracked.

Accordingly, it is a feature of the present invention to provide a method in which improved separation of anions using capillary electrophoresis is accomplished by precise control of the temperature of the capillary column. This, and other features and advantages of the present invention will become apparent from the following detailed description, the accompanying drawings, and the appended claims.

Brief Description of the Drawings

Figs. 1A - 1E are electropherograms of absorbance units versus time showing the effect of temperature on the separation by capillary electrophoresis of five anions;

Fig. 2 is a combined electropherogram showing the effect of temperatures ranging from 15°C to 60°C on the separation by capillary electrophoresis of five anions;

Fig. 3 is an electropherogram of absorbance units versus time for the separation by capillary electrophoresis of eight organic anions;

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Fig. 4 is an electropherogram of absorbance units versus time for the separation by capillary electrophoresis of five organic anions;

Fig. 5 is an electropherogram of absorbance units versus time for the separation by capillary electrophoresis of organic acids in white wine; and

Fig. 6 is an electropherogram of absorbance units versus time for the separation by capillary electrophoresis of organic acids in apple juice run against acids standards to illustrate the reproducibility of the process.

Detailed Description of the Preferred Embodiments

The present invention utilizes capillary electrophoresis in conjunction with precise temperature control to achieve improved separation and detection of common inorganic and organic anionic species. The preferred method of detecting such anions is the use of indirect photometric techniques using a UV/visible detector. For example, Foret et al., J. Chromatography, 470:299-308 (1989), describes one procedure for the indirect photometric detection of ions in capillary electrophoresis. In other embodiments of the invention, detection may be accomplished through the use of a conductivity detector or a mass spectrometer.

In a preferred embodiment of the invention using indirect photometric techniques, the process may be carried out manually by filling a capillary tube with a carrier electrolyte containing one or more light-absorbing co-anions of appropriate mobility. Typically, an untreated fused silica capillary tube having a length of 20-120 cm and an internal diameter of between about 25 to 250μ is preferred. The sample containing the anions of interest may then be introduced into the capillary using such standard techniques in the art as hydrostatic pressure, vacuum (hydrodynamic), or

electrokinetic injection in which the sample is moved into the capillary by an electrical potential.

After introduction of the sample, the ends of the capillary tube are immersed into liquid-containing reservoirs. The temperature of the capillary tube may be controlled by forced air or liquid circulating around the capillary or by placing the capillary between metal radiator plates. A detector is positioned downstream from the position where the sample is introduced into the capillary, and an electric current is applied to cause the ions in the sample to migrate past the detector. A preferred detector is one which utilizes UV/visible absorbance such as a multi-wavelength, scanning UV/VIS detector.

While a manual method may be used, a preferred method for separating and detecting the anions of interest in the present invention is to use an automated capillary electrophoresis instrument such as a SpectraPHORESIS® 1000 system commercially available from Spectra-Physics Analytical, Inc. of Fremont, California. Such a system includes an air controlled chamber in which the capillary is maintained at a precisely controlled temperature to within ±0.5°C, and preferably to within ±0.1°C. Operation of the thermal control for such a capillary electrophoresis instrument is described by Weinberger et al. in U.S. Patent No. 5,066,382, the disclosure of which is hereby incorporated by reference.

The present invention utilizes chemical compounds which facilitate detection of anions in a sample by indirect photometric techniques. Light-absorbing co-anions for use in the practice of the present invention include salts of anionic species known in the art to absorb visible or ultraviolet light. Such salts include chromates, vanadates, phthalates, pyromellitates, benzoates, and singly or multiply-charged carboxylates. For the detection and

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separation of inorganic anions such as chloride, sulfate, nitrite, and nitrate, preferred salts are potassium dichromate or potassium chromate and salts of 1,2,4,5 benzene tetracarboxylic acid (pyromellitic acid or PMA) having concentrations of from about 10μM to 20mM. For the detection and separation of organic anions such as tartrate, malate, succinate, lactate, acetate, propionate, butyrate, citrate, and caprylate, a preferred salt is potassium hydrogen phthalate having a concentration of from about 1 mM to 10 mM.

The method of the present invention may also be carried out by including an electroosmotic flow modifier in the carrier electrolyte which controls the speed and/or direction of the electroosmotic flow of the carrier electrolyte. While there are many known electroosmotic flow modifiers in the art, preferred electroosmotic flow modifiers for use in this invention include diethylenetriamine (DETA) and aliphatic trimethyl ammonium halides or hydroxides such as tetradecyltrimethylammonium bromide (TTAB). Preferred concentration ranges are from about 50 μM to 10 mM.

The present invention is particularly suited to detect such common low molecular weight inorganic and organic anions as chloride, nitrate, nitrite, sulfate, and oxalate. Many of these anions have been difficult to detect using conventional CZE techniques in the past because they do not absorb significant amounts of ultraviolet light. As these smaller anions have high mobilities in the carrier electrolyte, it is preferred that an electroosmotic flow modifier such as DETA be used to suppress (but not reverse) electroosmotic flow. Further, such smaller anions exhibit better peak shapes when the mobility of the light-absorbing co-anion approximates the mobility of such anions.

One preferred ultraviolet light-absorbing co-ion for use in separating and detecting smaller inorganic and organic anions is dichromate. Dichromate has a high mobility in an

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electric field and approximates the mobilities of the anions of interest. Further, dichromate strongly absorbs ultraviolet light. Another preferred ultraviolet light-absorbing co-ion for use in separating and detecting smaller anions is 1,2,4,5 benzene tetracarboxylic acid (PMA). PMA, with two more carboxylate groups than phthalate has a high mobility due to its higher charge density. We have found that the mobility of PMA is enhanced when the carrier electrolyte is at a pH of 6 or greater, where the acidic carboxylate groups are predominantly ionized.

As is known in this art, increasing the temperature in the capillary decreases the carrier electrolyte solution viscosity. This speeds the migration times of the anions past the detector. However, we have unexpectedly found that temperature also affects the selectivity (i.e., the order of migration) of the separation. Nitrate and nitrite ions migrate with the same relationship to each other, while chloride, sulfate, and oxalate ions also maintain their same relative relationship. However, the nitrogen-containing ions migrate relatively more slowly than the other ions as the temperature in the capillary is increased. Depending upon the target temperature selected, the elution order of the anions may be changed.

By precisely controlling temperature in the capillary, selectivity of the separation, as well as reproducibility, is controlled. The migration times for specific anions is thus predictable and reproducible from run to run to aid in the identification and quantification of such anions. Further, by varying the temperature during a run by increasing it, early peak resolution for highly mobile anions may be maintained while speeding the migration times of later, less mobile anion peaks.

We have also found that simultaneous monitoring by the detector at two different wavelengths provides an additional

means of identifying the anions of interest. The nitrogen-containing anions may be distinguished from other anions when absorption is simultaneously monitored at both 210 and 254 nm. For nitrate and nitrite, there is strong absorption at 210 nm but not at 254 nm so that a positive peak is observed at the lower wavelength, but not at the upper wavelength. The presence of positive peaks at 210 nm is thus an identifier of a nitrogen-containing anion in a sample. Additionally, the limits of detection are lower at the shorter wavelength for nitrate and nitrite anions (50 ppb at 210 nm). For other anions such as chloride and sulfate, limits of detection are lower at 254 nm (50 ppb). Thus, by simultaneously monitoring the sample at two different wavelengths, sensitivity of the process is enhanced.

In another embodiment of the invention, organic anions may be separated and detected using the same indirect photometric techniques. A preferred light-absorbing co-anion for use in separating and detecting organic anions is potassium acid phthalate. The use of phthalate as a co-anion in conjunction with an electroosmotic flow modifier such as TTAB, which reverses the electroosmotic flow of the sample, provides good resolution and peak shape of such common organic anions such as tartrate, malate, succinate, lactate, oxalate, acetate, propionate, butyrate, citrate, and caprylate.

In order that the invention may be more readily understood, reference is made to the following examples, which are intended to be illustrative of the invention, but are not intended to be limiting in scope.

Example 1

Detection and separation of several common anions was carried out using capillary electrophoresis with a SpectraPHORESIS® 1000 system (Spectra-Physics Analytical,

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Inc. of Fremont, California). The capillary was untreated, fused silica, 70 cm in length and with a 75μ internal diameter. A carrier electrolyte solution was prepared as follows. A first stock solution was prepared by dissolving 160 mM potassium dichromate and 1.3 mM H_2SO_4 in deionized water. A second stock solution was prepared by dissolving boric acid (H_3BO_3) and sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) in deionized water to a concentration of 2.4 M borate; 50 mM of DETA was also added.

The two stock solutions were then combined and diluted in deionized water to a final concentration of 48 mM borate, 1 mM DETA, 1.8 mM dichromate at pH of 8.0. In order to maintain the oxidation state of the dichromate, the stock solutions were not combined until the day of use. Stock solutions of 1000 ppm chloride, nitrate, nitrite, sulfate, and oxalate anions were also prepared by dissolving NaCl, Na_2SO_4, $NaNO_3$, $NaNO_2$, and $H_2C_2O_4 \cdot 2H_2O$ in deionized water. The anion solutions were diluted as required.

Injection of the sample was carried out using electrokinetic injection for 5 sec at -10kV. Capillary electrophoresis of individual samples was carried out at -30kV at temperatures of 20°C to 60°C, in 10°C intervals. This operation using a borate buffer produced only a 40 μamp current, permitting enhanced sensitivity for the system. The temperature in the capillary was controlled to within ± 0.1°C of the target temperature for each sample run. The UV/VIS scanning detector on the system was operated to monitor at 210 nm and 254 nm, simultaneously.

As can be seen from Figs. 1A - 1E, the temperature at which the process is carried out is crucial to the selectivity of the separation of the anions. Further, the monitoring of the separation at both 210 nm and 254 nm shows the positive nitrite and nitrate peaks which permits easy identification of these anions. The sulfate-oxalate pair

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migrates relatively faster than the nitrate-nitrite pair as the temperature is increased. At 20°C, sulfate and oxalate are the last anions to pass the detector. At 30°C and 50°C, two ions co-elute so that only four peaks are distinguished (at 30°C, nitrate and sulfate co-elute; at 50°C, nitrite and sulfate co-elute). At 60°C, sulfate precedes the nitrogen-containing ions and oxalate appears between them.

The optimal temperature for this separation was 40°C, which takes advantage of both the rapid cycle time and ease of identification of peaks when both 210 nm and 254 nm are monitored.

Example 2

Using the same equipment as in Example 1, the same five anions, chloride, sulfate, nitrate, nitrite, and oxalate, were separated and detected using a carrier electrolyte containing 7 mM PMA as the light-absorbing co-anion, 7 mM sodium hydroxide, and 2 mM DETA at a pH of 9.6. The sodium hydroxide was used to adjust the pH. Mobility of PMA was enhanced at pH 9.6 due to ionization of all carboxylate groups.

Sample injection was carried out using electrokinetic injection for 2 sec at -5kV. Capillary electrophoresis of individual samples was carried out at -25kV at temperatures of 15°C, 17.5°C, 20°C, 25°C, 30°C, 40°C, 50°C, and 60°C, respectively. The current was less than 40μamp using a 35 cm/50μm internal diameter untreated, fused silica capillary. The separation was monitored at 254 nm. The results of the separations are shown in Fig. 2.

Again, selectivity of the separation is dependent upon temperature. Nitrite and nitrate ions migrate with the same relationship to each other, while chloride, sulfate, and oxalate also maintain their same relative distances. However, because the nitrogen-containing anions migrate relatively more slowly than the other anions as the

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temperature is increased, the elution order is a function of the temperature in the capillary. The optimal temperature for this separation using PMA as the UV absorbing co-anion was 60°C which produced separate, distinct peaks for each anion.

Example 3

Using the same equipment as in Example 1, a number of organic anions were separated and detected. A carrier electrolyte concentrate was prepared by dissolving 50 mM potassium hydrogen phthalate, 5 mM TTAB, and 500 mM 2-(N-morpholino)-ethanesulfonic acid (MES) in deionized water. pH was adjusted to 5.2 using NaOH. When diluted 1:10, the carrier electrolyte had a final concentration of 5 mM phthalate, 0.5 mM TTAB, and 50 mM MES. Stock solutions of 10 ppm organic anions were prepared by dissolving oxalic acid, L-tartaric acid, D,L-malic acid, succinic acid, propionic acid, caprylic acid, and n-butyric acid (sodium salt) in separate deionized water samples.

Sample injection was carried out using electrokinetic injection for 1 sec at -10kV. Capillary electrophoresis of individual samples was carried out at -30kV at a temperature of 20°C. The current was less than 20μamp using a 70 cm in length and 75 μm internal diameter untreated, fused silica capillary. The separation was monitored at 205 or 210 nm. The results of the separations, which were run at a pH of 5.0 in the absence of MES, are shown in Fig. 3. The elution order was oxalate, tartrate, malate, succinate, lactate, acetate, propionate, and butyrate. The inclusion of 50 mM of Good's buffer (MES) provided stabilization against pH changes as well as an improved baseline and better peak shape as shown in Fig. 4. The addition of TTAB reversed the

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electroosmotic flow. The elution order of the anions was: oxalate, tartrate, malate, succinate, and lactate.

The presence of these organic acids can be detected in beverages such as white wine and apple juice as shown in Figs. 5 and 6, respectively. Acetate is a major component of this wine as shown in Fig. 5. Fig. 6 also demonstrates the reproducibility of this process run against acids standards as the malate negative peak appears at about 3 min. in both the apple juice and the acids standard.

While certain representative embodiments and details have been shown for purposes of illustrating the invention, it will be apparent to those skilled in the art that various changes in the methods and apparatus disclosed herein may be made without departing from the scope of the invention, which is defined in the appended claims.

What is claimed is:

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1. A method for detecting and separating anions in a sample using capillary electrophoresis comprising the steps of, providing a capillary filled with a carrier electrolyte, heating or cooling said capillary to a target temperature different from ambient temperature, introducing a sample containing one or more anions into said capillary, applying an electrical current to said capillary under conditions causing anions in said sample to migrate and separate, and detecting said anions while maintaining the temperature in said capillary to within ±0.5°C of said target temperature.

2. The method of claim 1 in which said carrier electrolyte contains a light-absorbing co-anion, and said anions are detected indirectly using a photometric detector.

3. The method of claim 1 in which said target temperature is in the range of from 25°C to 60°C.

4. The method of claim 1 in which said target temperature is ~~10°C.~~ 40°C.

5. The method of claim 1 in which said target temperature is varied as said anions migrate.

6. The method of claim 1 in which said sample contains one or more anions selected from the group consisting of chloride, nitrate, nitrite, sulfate, and oxalate anions.

7. The method of claim 1 in which said one or more anions are detected by simultaneously monitoring said sample at two different wavelengths.

8. The method of claim 7 in which said sample is monitored at 205 or 210 and 254 nm simultaneously.

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9. The method of claim 1 including the step of including an electroosmotic flow modifier in said carrier electrolyte.

10. The method of claim 9 in which said electroosmotic flow modifier is diethylenetriamine.

11. The method of claim 9 in which said electroosmotic flow modifier is tetradecyltrimethylammonium bromide.

12. The method of claim 1 in which said sample contains one or more anions selected from the group consisting of oxalate, tartrate, malate, succinate, lactate, ~~acetate~~, propionate, butyrate, citrate, and caprylate.

13. The method of claim 12 including tetradecyltrimethylammonium bromide as an electroosmotic flow modifier.

14. A method for detecting and separating anions in a sample using capillary electrophoresis comprising the steps of, providing a capillary filled with a carrier electrolyte, heating said capillary to a target temperature in the range of from 20° to 60°C, introducing a sample containing one or more anions selected from the group consisting of chloride, nitrate, nitrite, sulfate, and oxalate anions into said capillary, applying an electrical current to said capillary under conditions causing anions in said sample to migrate and separate, and detecting said anions by simultaneously monitoring said sample at two different wavelengths while maintaining the temperature in said capillary to within ±0.5°C of said target temperature.

12 15. The method of claim 11 in which said carrier electrolyte contains a light-absorbing co-anion, and said anions are detected indirectly using a photometric detector.

13 16. The method of claim 12 in which said sample is monitored at 205 or 210 and 254 nm simultaneously.

15 17. The method of claim 11 including the step of including an electroosmotic flow modifier in said carrier electrolyte.

16 18. The method of claim 15 in which said electroosmotic flow modifier is diethylenetriamine.

19. The method of claim 11 including the step of introducing a second portion of said sample into said capillary containing one or more anions, changing said temperature in said capillary, detecting said anions in said second portion, and comparing the order of elution of said anions in said second portion with said first portion.

19

Abstract of the Disclosure

An improved method for the separation of anions using capillary electrophoresis techniques. Both organic and inorganic anions may be separated. Using precise control of the temperature of the fluid in the capillary column, the migration speed and order of migration of the anions may be controlled to improve the selectivity of the process. Further, close temperature control provides a high degree of repeatability for samples and enables one to track and identify specific anions.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the invention entitled:

CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS, described and claimed

__X__ in the attached specification;
_____ in the specification filed _____,
as U.S. Application Serial No. _____, and as amended _____.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims as filed and as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56(a).

I appoint

	Richard A. Killworth	Reg. No. 26,397
	James F. Gottman	Reg. No. 27,262
6	Timothy W. Hagan	Reg. No. 29,001
	Richard C. Stevens	Reg. No. 28,046
	Robert L. Showalter	Reg. No. 33,579
	Scot R. Hewitt	Reg. No. 35,191

my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Address all telephone calls to (513) 223-2050. Address all correspondence to: KILLWORTH, GOTTMAN, HAGAN & SCHAEFF, 1400 One First National Plaza, Dayton, Ohio 45402-1502.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1-00

Full name of sole or first Inventor: Lenore Kelly

Inventor's signature Lenore Kelly

Date: July 02, 1993

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Citizenship: USA

Post Office Address: c/o Thermo Separation Products
(California) Inc.
P.O. Box 5116
Fremont, CA 94537

2-00

Full name of second Inventor: Dean S. Burgi

Inventor's signature

Date: 7/2/93

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Citizenship: USA

Post Office Address: c/o Thermo Separation Products
(California) Inc.
P.O. Box 5116
Fremont, CA 94537

3-00

Full name of third Inventor: Robert John Nelson

Inventor's signature

Date: 2 July 1993

Residence: 45757 Northport Loop West CA
Fremont, CA 94537

Citizenship: USA

Post Office Address: c/o Thermo Separation Products
(California) Inc.
P.O. Box 5116
Fremont, CA 94537

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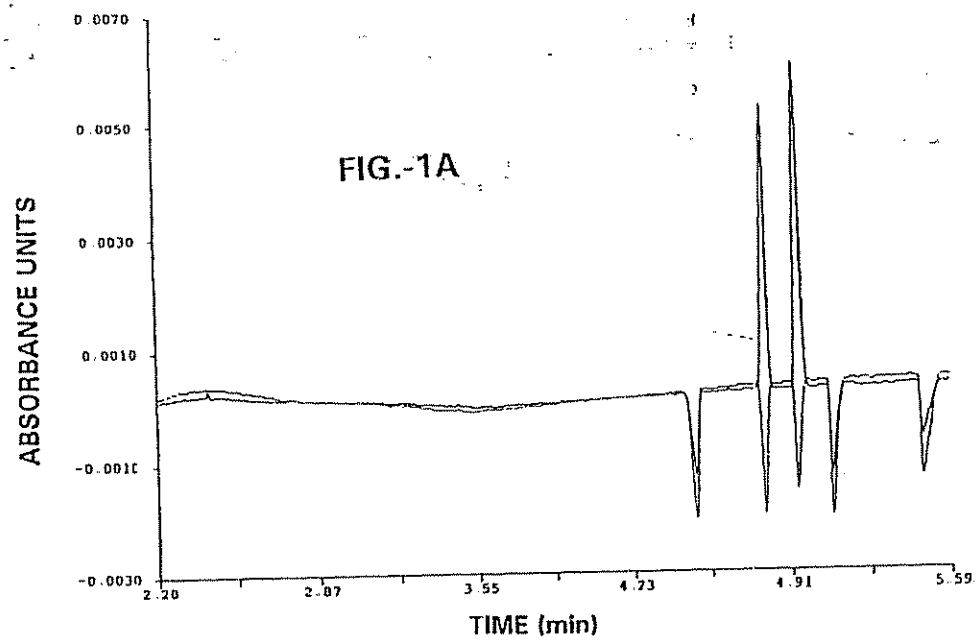
FIG.-1A
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-0.0010
-0.0030
2.20
2.87
3.55
4.23
4.91
5.59
TIME (min)

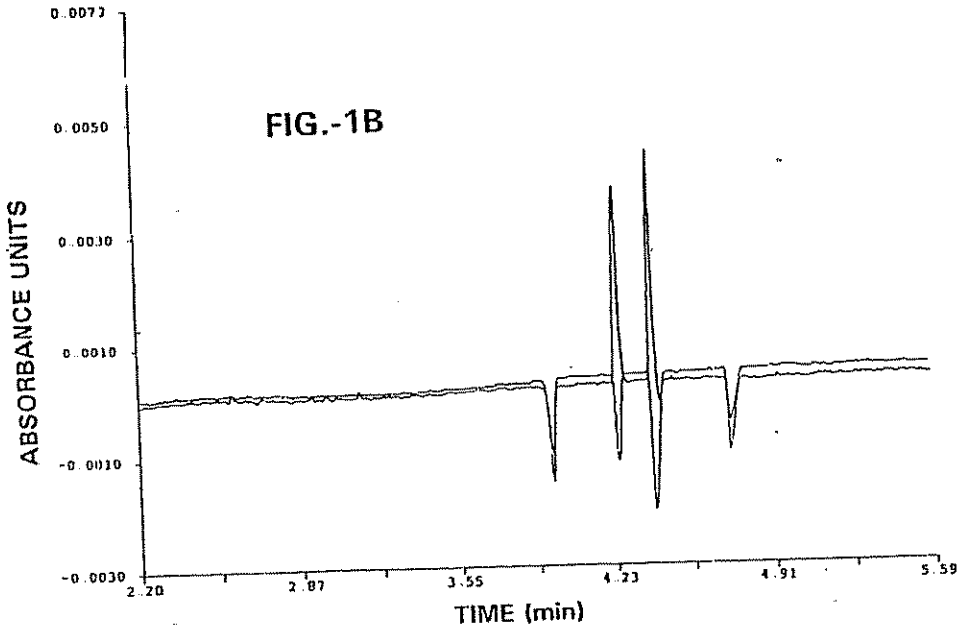
FIG.-1B
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0.0030
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-0.0030
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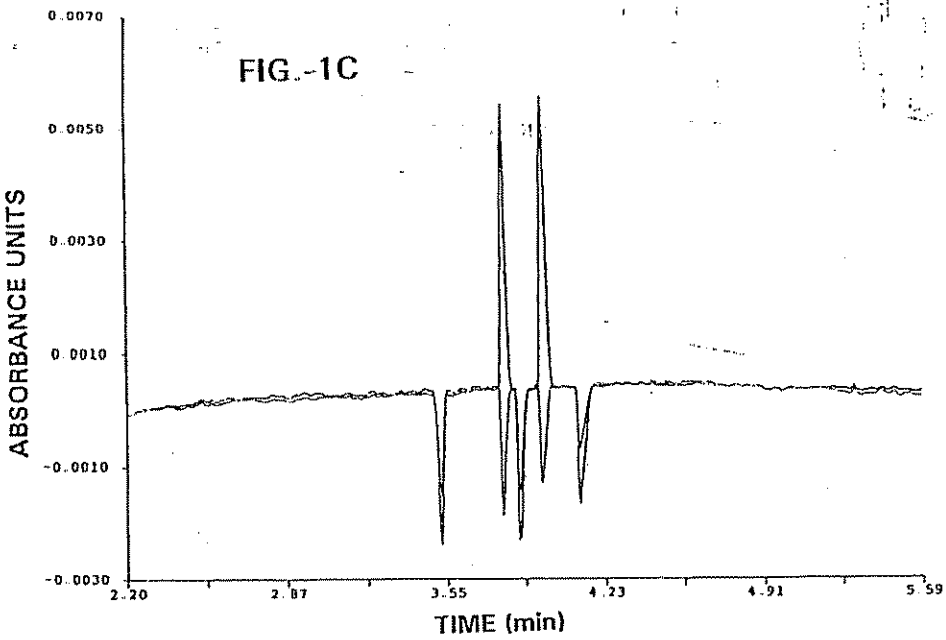
4.23
4.91
5.59
TIME (min)

FIG.-1C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0.0050
0.0030
0.0010
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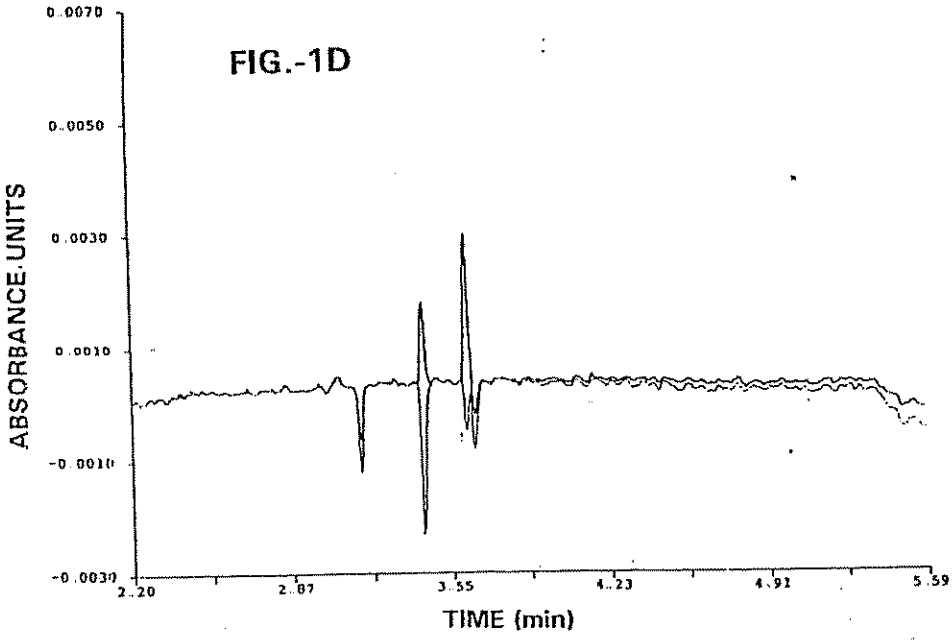
-0.0030
ABSORBANCE UNITS
2.20
2.87
3.55
4.23
4.91
5.59
TIME (min)

FIG.-1D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0.0010
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ABSORBANCE UNITS
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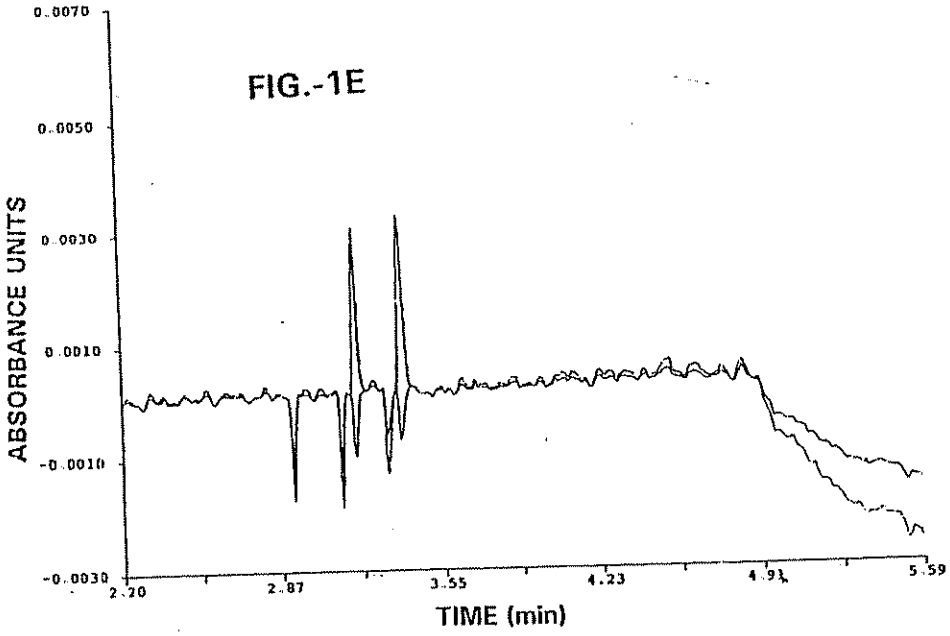
2.87
3.55
4.23
4.91
5.59
TIME (min)

FIG.-1E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0.0050
0.0030
0.0010
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ABSORBANCE UNITS
2.20
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3.55
4.23
4.91
5.59
TIME (min)

FIG. 2

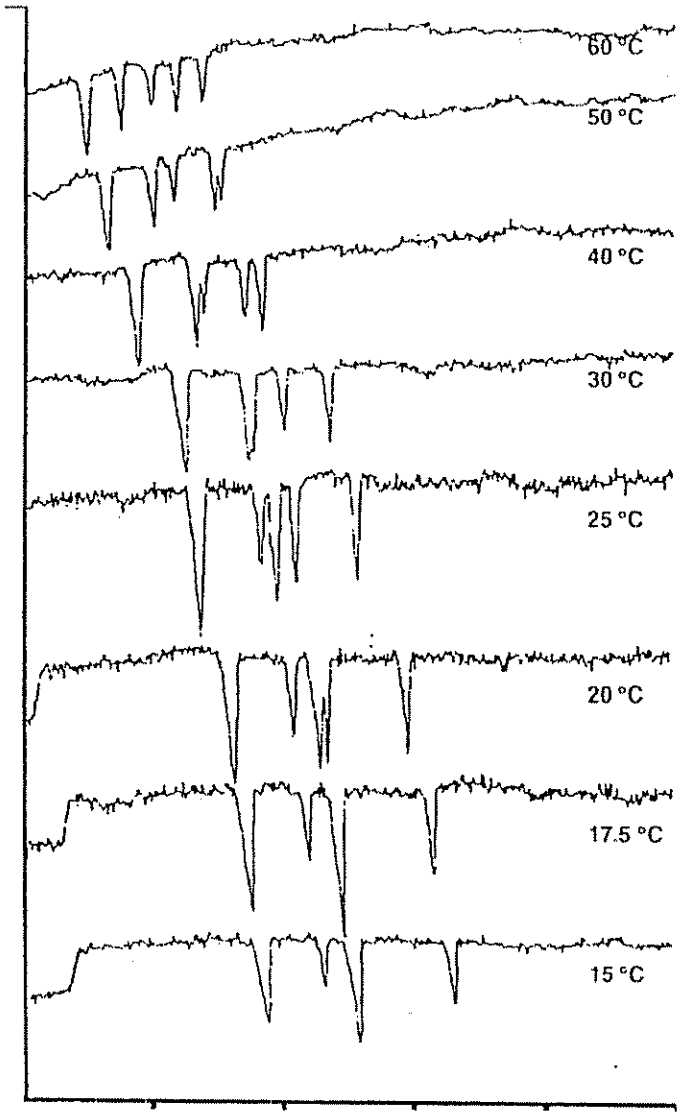
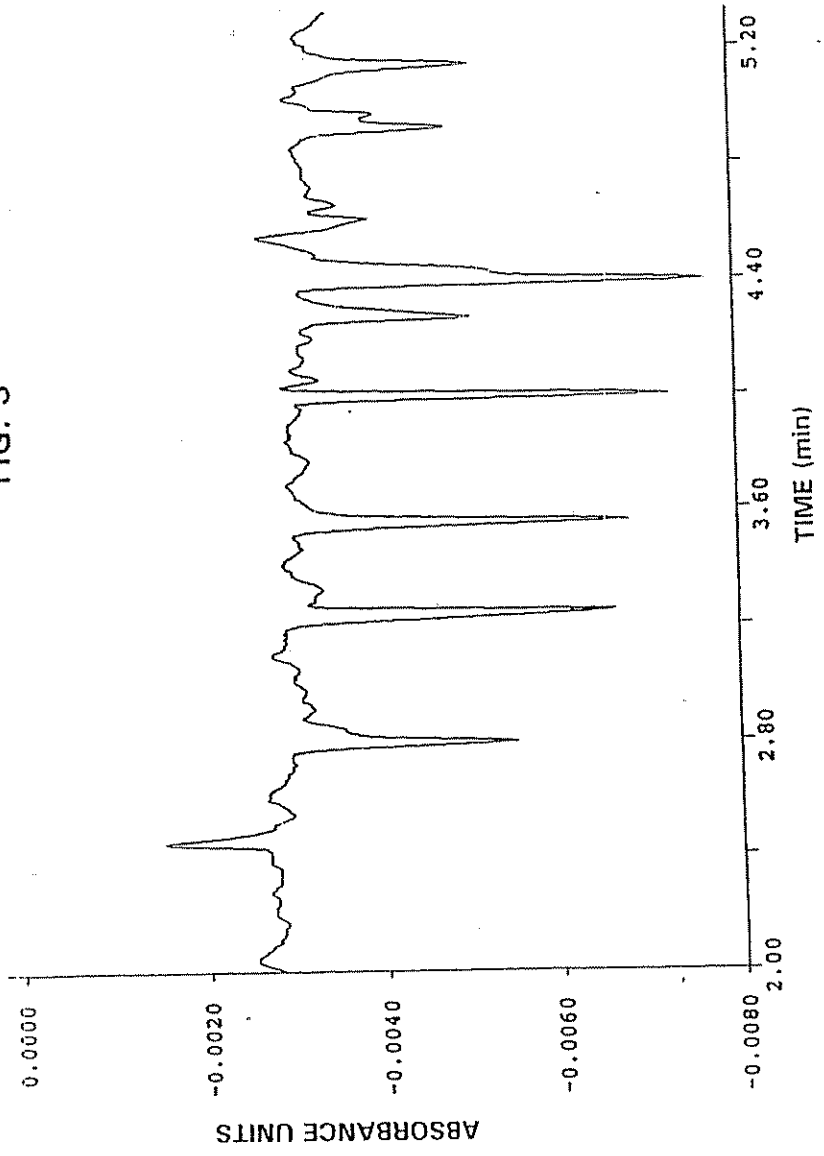


FIG. 3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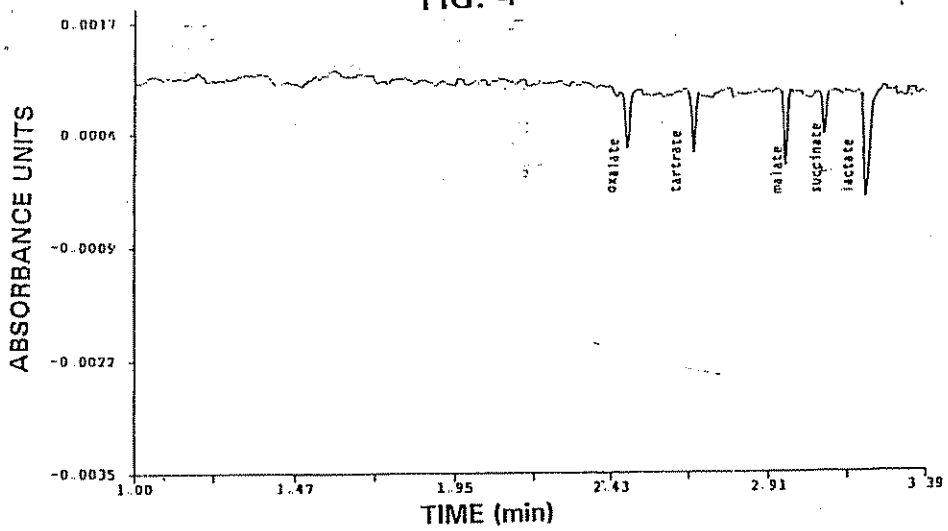
2.00
2.80
3.60
4.40
5.20
TIME (min)

FIG. 4


ABSORBANCE UNITS
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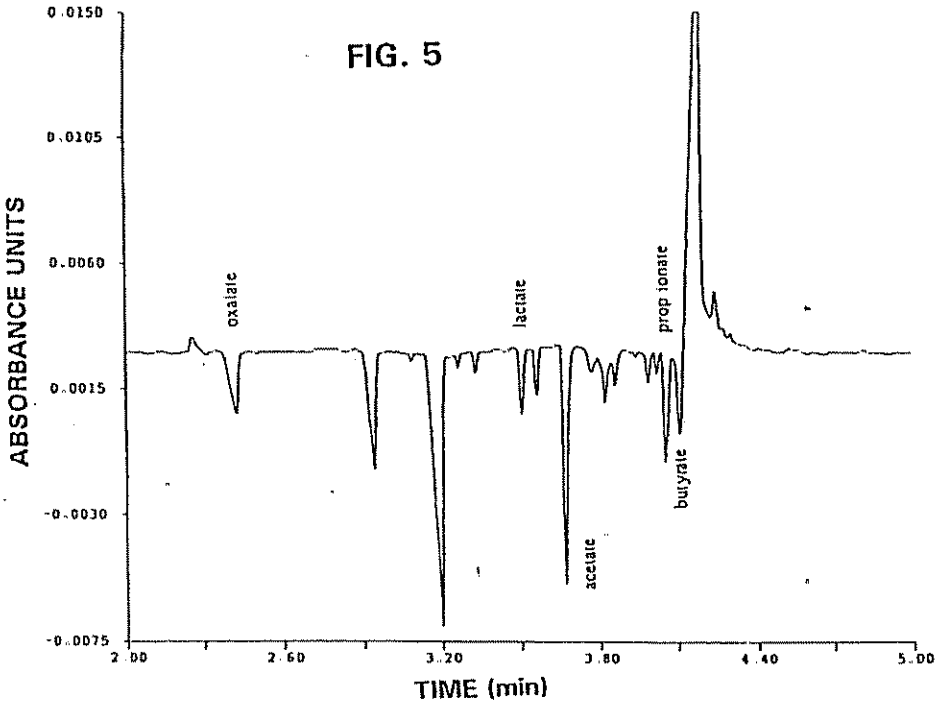
0.0004
-0.0009
-0.0022
-0.0035
oxalate
tartrate
malate
succinate
lactate
1.00
1.47
1.95
2.43
2.91
3.39
TIME (min)

FIG. 5


ABSORBANCE UNITS
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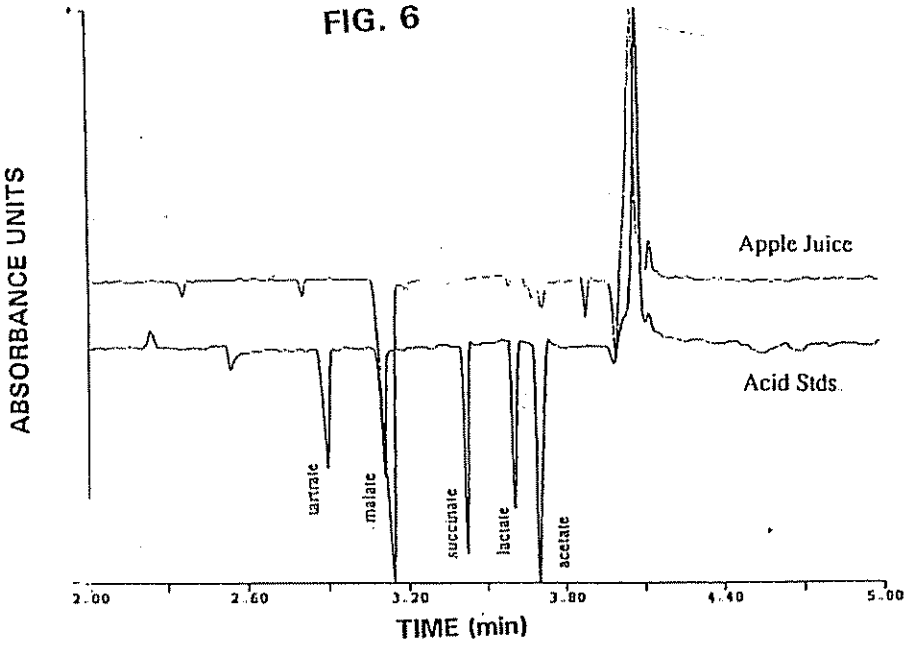
0.0060
0.0015
-0.0030
-0.0075
oxalate
lactate
acetate
propionate
butyrate
2.00
2.60
3.20
3.80
4.40
5.00
TIME (min)

FIG. 6


ABSORBANCE UNITS
Apple Juice
Acid Stds.
tartrate
malate
succinate
lactate
acetate
2.00
2.60
3.20
3.80
4.40
5.00
TIME (min)

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08/088439

FIG.-1A

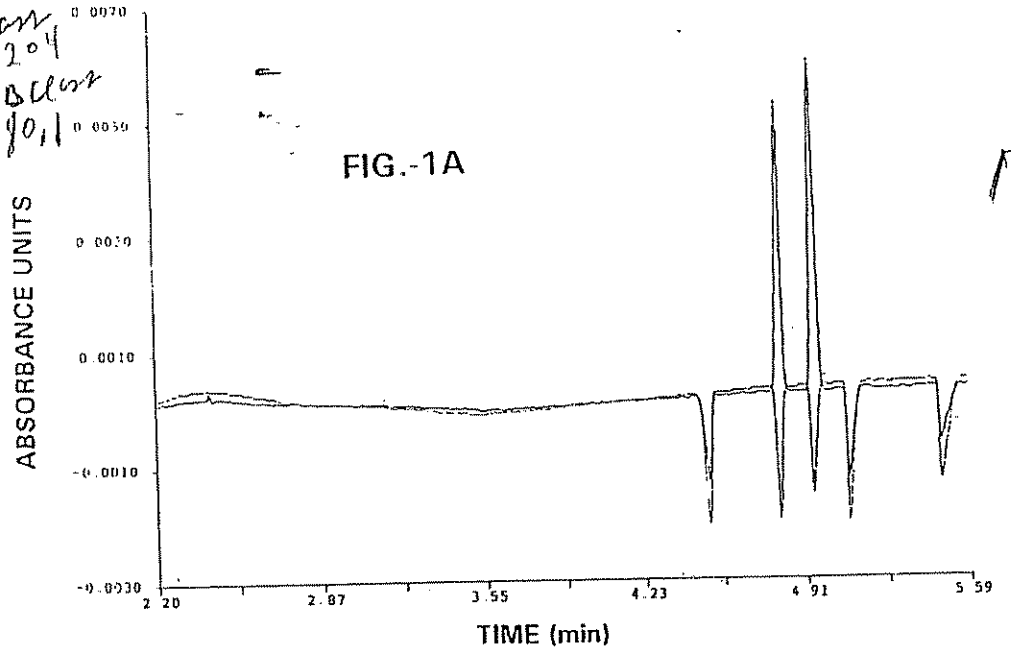

FIG.-1B

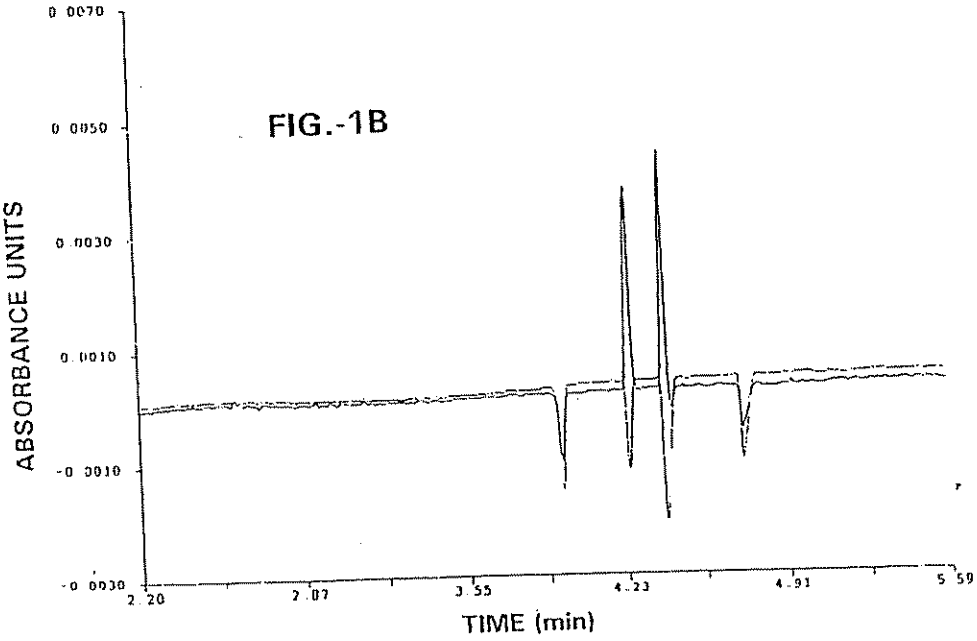

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FIG.-1C

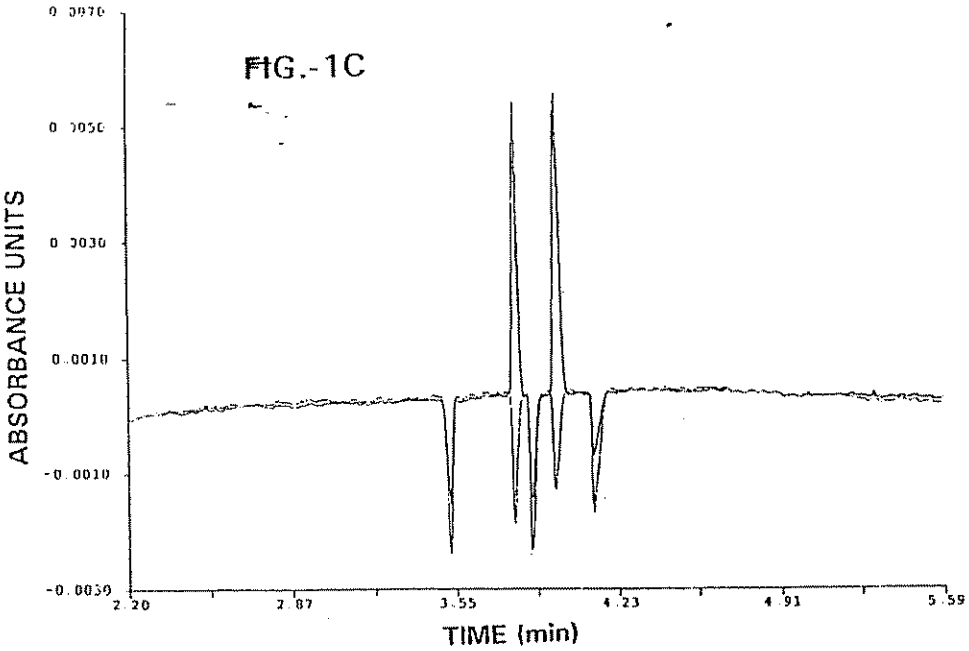

FIG.-1D

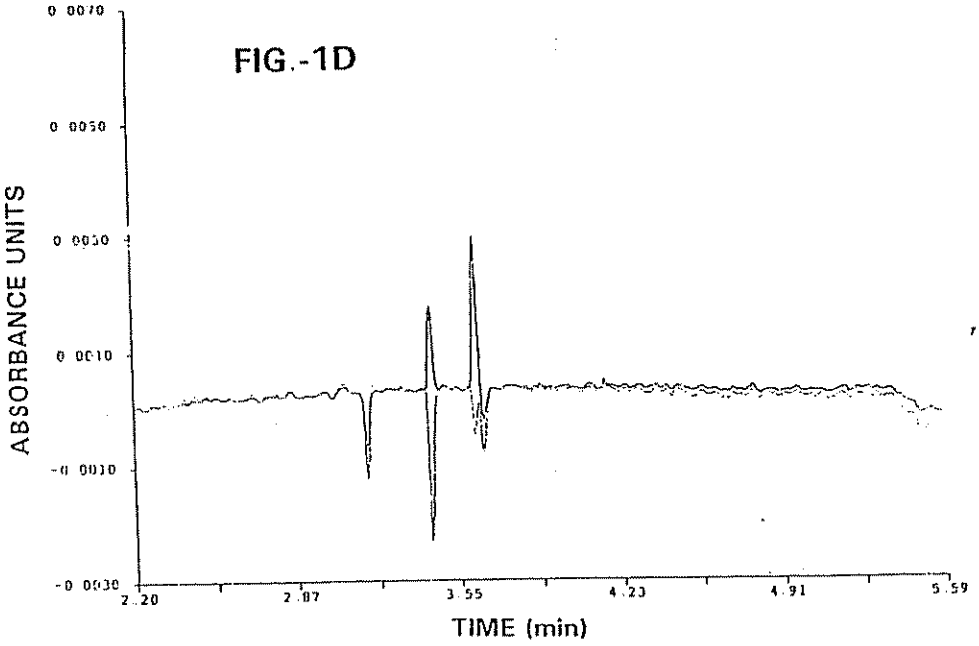

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FIG.-1E

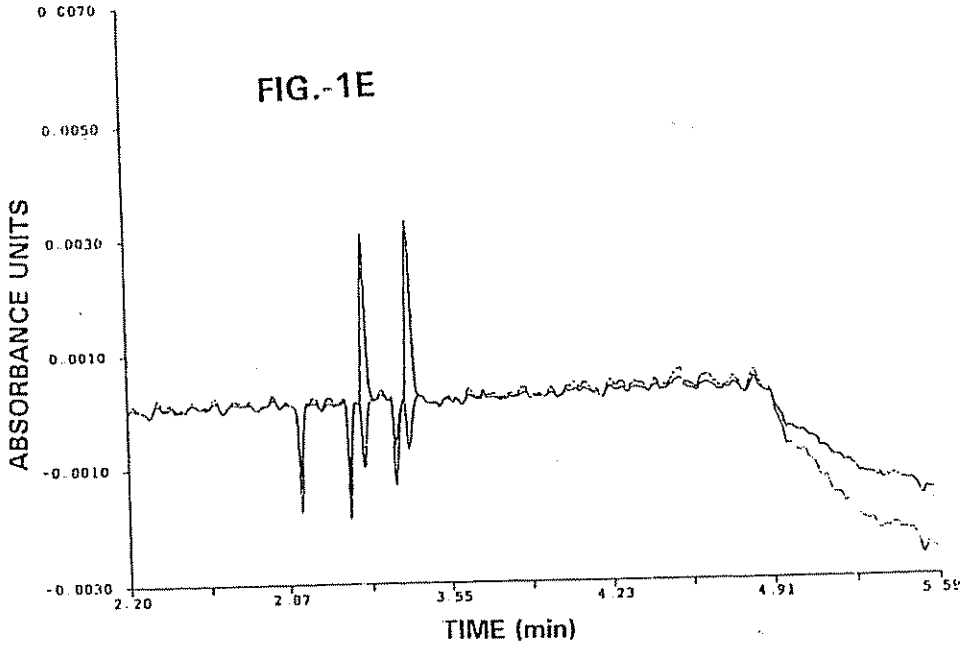

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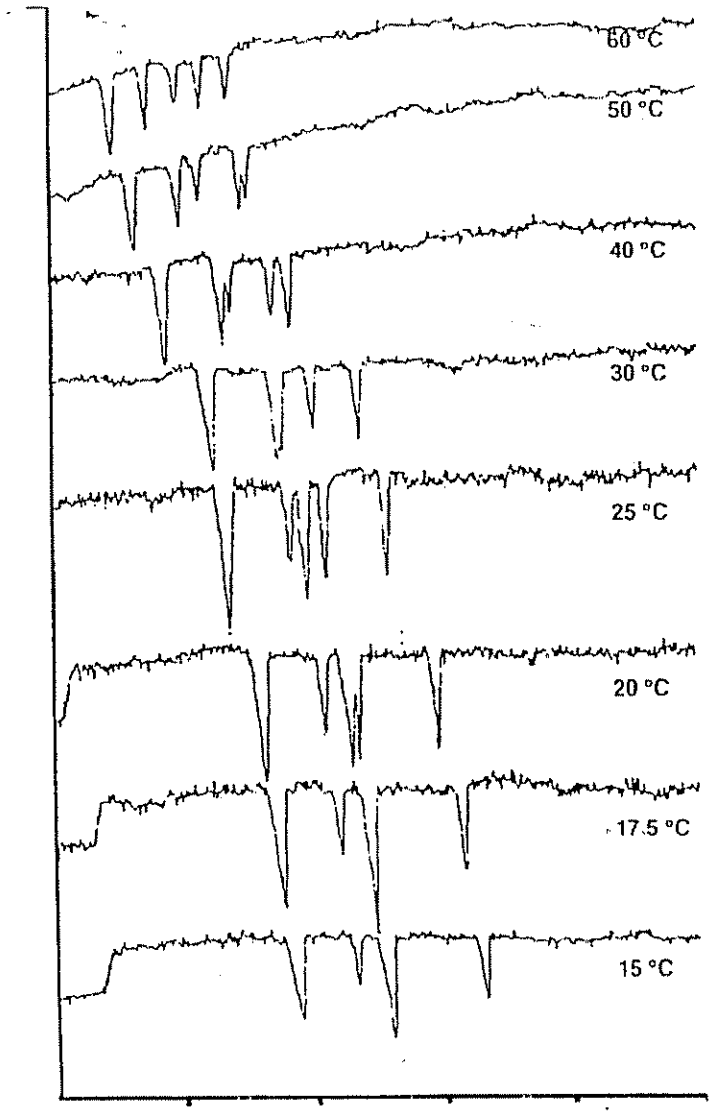
FIG. 2



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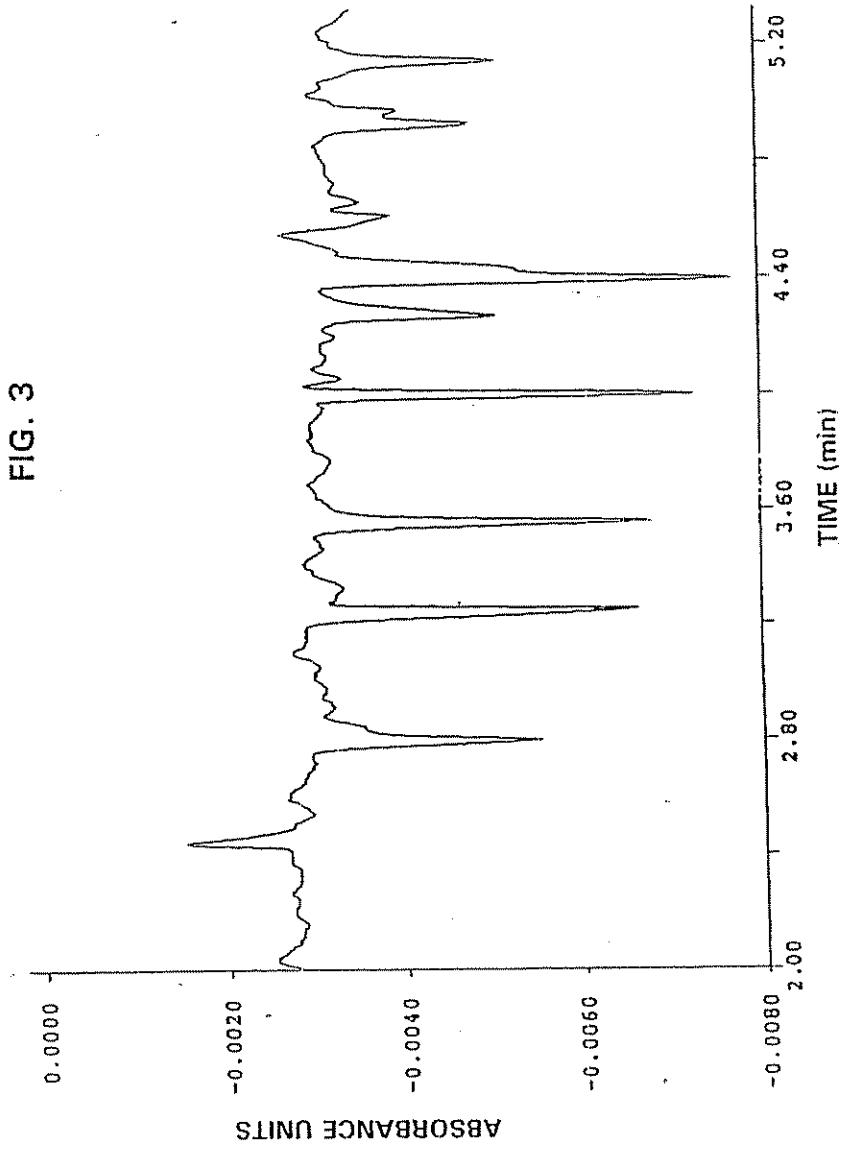

FIG. 3

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FIG. 4

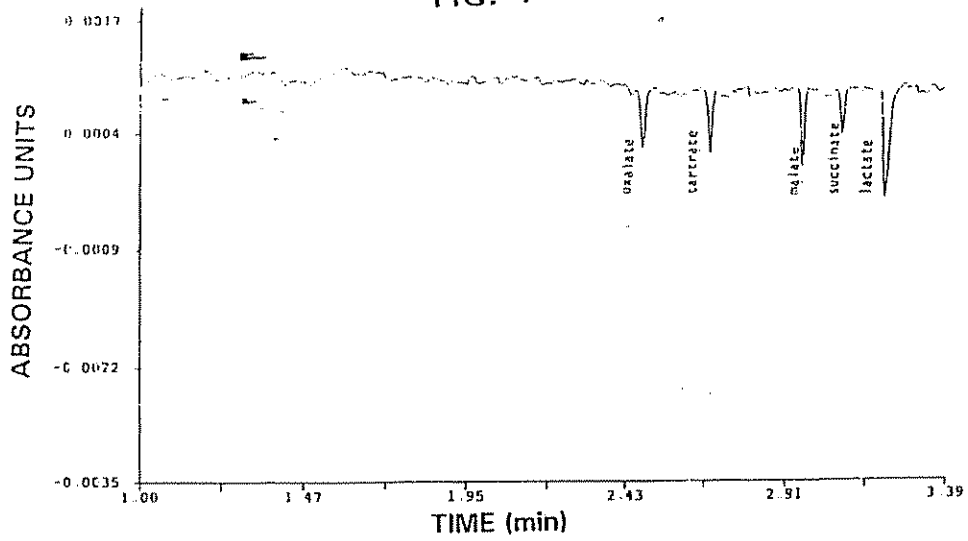

FIG. 5

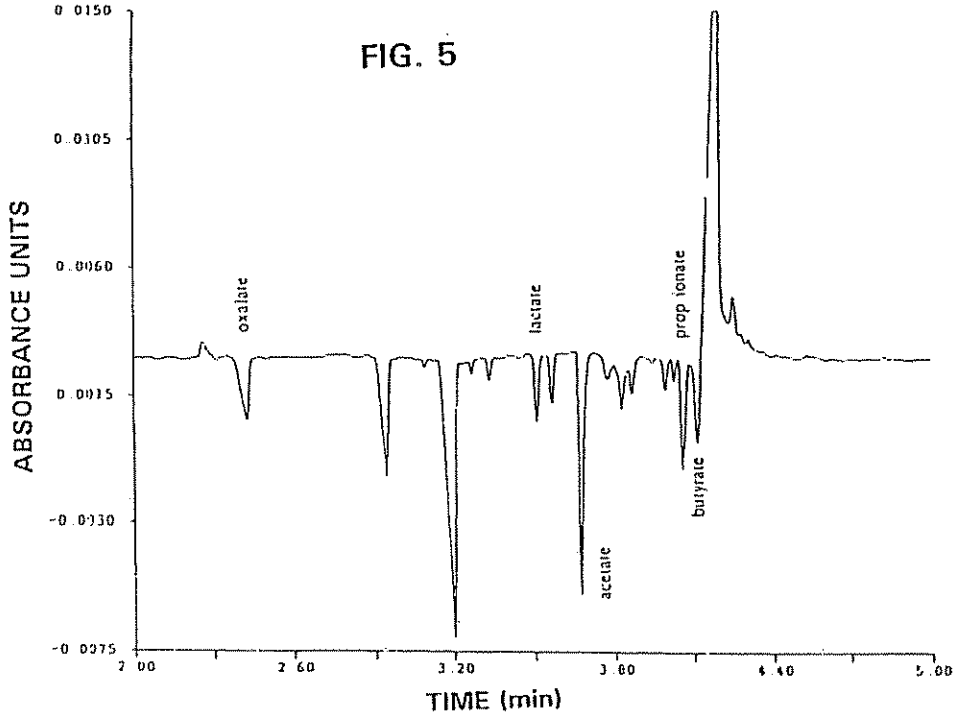

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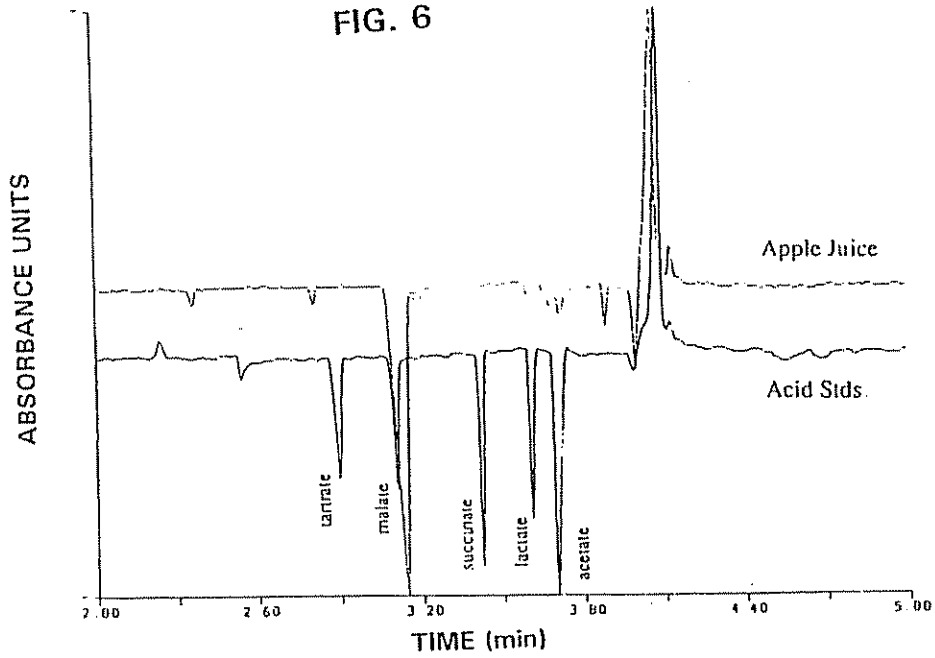
FIG. 6



UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/088,439	07/07/93	KELLY L.	SPA096PA

EXAMINER
STARSIAK, J

D1M1/1020
KILLWORTH, GOTTMAN, HAGAN & SCHAEFF
1400 ONE FIRST NATIONAL PLAZA
DAYTON, OH 45402-1502

ART UNIT	PAPER NUMBER
1102	2

DATE MAILED: 10/20/93

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☒ This application has been examined ☐ Responsive to communication filed on _____ ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), _____ days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-19 are pending in the application.
   Of the above, claims _____ are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 1-4, 6, 7, 9, 11-15, and 17 are rejected.
5. ☒ Claims 5, 8, 10, 16, 18, and 19 are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings ☐ are ☐ acceptable. ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed on _____ has been ☐ approved. ☐ disapproved (see explanation).
12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____
13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 9-80)

Claims 1-12 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

In claim 1, the applicant recites heating or cooling said capillary to a target temperature different from ambient temperature". This recitation is indefinite because use ambient temperature is not fixed". In fact in claim 3, which depends from claim 1, the applicant recites, "said target temperature is in the range of from 25°C to 60°C". 25°C (77°F) could easily be the ambient temperature of a laboratory. The remaining claims are rejected because they depend on claim 1.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 3, and 4 are rejected under 35 U.S.C. § 102(b) as being clearly anticipated by Morin et al.

Morin et al. discloses a method of capillary zone electrophoresis of the following anions: arsenite, arsenate, monomethylarsonic acid, and dimethylarsinic acid (see Summary). "The column temperature was set at 40°C" (see Experimental).

Claims 1, 2, 6, 7, 11, 12, 13, 14, 15, and 17 are rejected under 35 U.S.C. § 102(b) as being clearly anticipated by Jones et al.

Jones et al discloses method "particularly useful for the separating and detecting anions" (col. 2, lines 19-20). Said method "provides a light absorbing background at a wavelength suitable for sensitive and interference-free indirect photometric detection of all ionic species... In addition an alkyl quaternary ammonium salt with a least four carbon atoms in a linear or branched configuration is required ... tetradecyltrimethyl ammonium bromide (TTAB) or cetyltrimethyl ammonium bromide (CTAB) are particularly preferred quaternary ammonium salts (col. 2, line 24-36)". "Detection was carried out using a Linear Instruments variable UV/Vis CE detector at two different wavelengths: 254nm and 272nm" (col. 5, lines 62-64). Most of the anions recited in claims 5 and 12 were separated in examples 3 and 4 of Jones et al. Since Jones et al is silent concerning the temperature, it is presumed that temperature was at ambient temperature. Hence the temperature range 20° to 60°C is inherently met by Jones et al.

The following is a quotation of 35 U.S.C. § 103 which forms the basis for all obviousness rejections set forth in this Office action:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.
Subject matter developed by another person, which qualifies as prior art only under subsection (f) or (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

Evaluations of the level of ordinary skill in the art requires consideration of such factors as various prior art approaches, types of problems encountered in the art, rapidity with which innovations are made, sophistication of technology involved, educational background of those actively working in the field, commercial success, and failure of others.

The "person having ordinary skill" in this art has the capability of understanding the scientific and engineering principles applicable to the claimed invention. The evidence of record including the references and/or the admissions are considered to reasonably reflect this level of skill.

Claims 1, 2, 6, 7, 9, 11, 12, 13, 14, 15, and 17 are rejected under 35 U.S.C. § 103 as being unpatentable over Jones et al. in view of Morin et al.

For the details of Jones et al see 102 rejection above. The only difference between the claims and Jones et al is that Jones et al does not explicitly recite maintaining the capillary at a target temperature.

Morin et al. teaches that the temperature can effect the quality of the separation of anions using capillary zone electrophoresis and that how to maintain the capillary at a specific temperature is known in the art.

It would have been obvious to one having ordinary skill in the art at the time the invention was made to optimize the temperature used when performing the method of Jones et al because the criticality of this parameter is known in the art.

Claims 16, 18 and 19 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Claims 5, 8 and 16 are would be allowable if rewritten to overcome the rejection under 35 U.S.C. § 112 and to include all of the limitations of the base claim and any intervening claims.

An appropriate search of the prior art failed to reveal any reference which explicitly teaches or failing suggest a method of separating anion using capillary zone electrophoresis with one of the following features: 1. the temperature of the capillary is varied during the separation; 2. the sample is monitored at 205 or 210 and 254 nm simultaneously; 3. the electroosmotic flow modifier is diethylenetrianline; 4. introducing a second portion

of said sample into said capillary containing one or more anions, changing the temperature in the capillary, detecting anions in said second portion, and comparing the order of elution of said anions in said second portion with said first portion.

The prior art made of record and not relied upon is considered pertinent to applicant's disclosure.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to J. Starsiak Jr. whose telephone number is (703) 308-1797.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0661.

J. Starsiak:rg
October 07, 1993

John Niebling
Supervisory Patent Examiner
Patent Examining Group 110

TO SEPARATE, FOLD TOP AND BOTTOM EDGES, SNAP APART AND REMOVE CARBON

FORM PTO-892 (REV. 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO. 08/088,439	GROUP ART UNIT 1102	ATTACHMENT TO PAPER NUMBER 2
NOTICE OF REFERENCES CITED	APPLICANT(S) Lenore Kelly et al		

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A	3932264	1-1976	Haruki et al	204	299R	
	B	3941678	3-1976	Akiyama	204	299R	
	C	5104506	4-1992	Jones et al	204	180.1	
	D	5240576	8-1993	Lauer	204	180.1	2-1988
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R P. Martin, M B. Amran, S. Favier, R. Heimburger, and M. Leroy "Separation of arsenic anions by capillary zone electrophoresis with UV detection" Fresen-

S ius' Journal of Chemistry 342 (1992) 357-362

T Yasuyuki Kuroso, Kiyokatsu Hibi, Toru Sasaki, and Munao Saito,

U "Influence of Temperature Control in Capillary Electrophoresis" Journal of High Resolution Chromatography Vol. 14 (1991) 200-203

EXAMINER	DATE
John S. Starsiak, Jr.	Sept. 27, 1993

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

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Fresenius' Journal of

Fresenius J Anal Chem (1992) 342:357–362

Separation of arsenic anions by capillary zone electrophoresis with UV detection

P. Morin, M. B. Amran, S. Favier, R. Helmburger, and M. Leroy

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Summary. Capillary zone electrophoresis (CZE) in capillary silica columns has been used for the separation of arsenite (AsO_2^-), arsenate (AsO_4^{3-}), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). The separation of these ionic species has been achieved using a capillary silica column (72 cm × 50 μm i.d.) with an acidic phosphate buffer and with an on-column UV detection (190 nm). Optimization of experimental parameters (pH, temperature, voltage) were studied. The selectivity of the separation can be improved by working in the pH-range of 4.5–6.5. For analytical inorganic separations of UV-absorbing anions, capillary zone electrophoresis has advantages because of the relatively simple equipment, the short analysis time (15 min), the high efficiency and the low mass detection limit (40 pg for arsenate).

Introduction

Arsenic is present in both marine and freshwater organisms in the form of water-soluble and lipid-soluble compounds [1]. The level of total As in sea water is approximately 2 μg/kg [2, 3]. Four different arsenic species, arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), have been detected in sea water [4]. Analysis of marine algae has shown that arsenic is present in other forms than inorganic or methylarsenic compounds [5]. Several papers describe the speciation analysis of arsenic by ion chromatography [6–11] or reversed-phase liquid chromatography [12–17]. An alternative analytical method is capillary electrophoresis in which the analyte is introduced into a capillary tube and subjected to electrokinetic separation. Extensive reviews of capillary electrophoresis have been recently published in different journals [18–22]. Capillary electrophoresis (CE) is a very powerful separation technique for the separation of ionic as well as neutral substances. Narrow diameter capillary (50–75 μm i.d.) leads to an efficient dissipation of heat and suppresses convection, whereas the high voltage causes fast separations with high efficiency (10^5–10^6 theoretical plates within 30 min). The separation mechanism in free solution capillary electrophoresis (FSCE) is based on differences in the electrophoretic mobilities of species. These parameters depend on the structural differences of the solutes (size, shape and charge) and are also affected by the physico-chemical properties of the carrier electrolyte, such as pH, ionic strength, viscosity and dielectric constant. Many ions, such as iodate and periodate [23], metal ions [24] and hexacyanoferrate(II) and -(III) ions [25] can be separated by FSCE.

In this paper, the separation of arsenic species by capillary electrophoresis with an on-column UV detection is presented.

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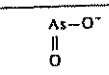
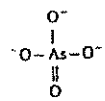
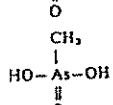
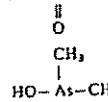
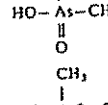
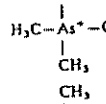
Experimental

Capillary electrophoresis. An Applied Biosystems (Foster City, CA, USA) Model 270A capillary electrophoresis system with a fused silica column was used for this work. A 72 cm × 50 μm i.d. silica capillary filled with phosphate buffer served as the separation tube operated at high voltage (10–40 kV). A small section of the polyimide coating of the capillary column was removed prior to filling to get an optical window for UV detection. Between analyses, the capillary was flushed with the buffer solution for 15 min. The on-column detection was operated at 190 nm at an absorbance range of 0.01 AUFS and a rise time of 1 s. The column temperature was set at 40° C. Samples were injected by the vacuum technique. All electropherograms were recorded using a Spectra Physics (San José, CA, USA) Chromjet Model integrator.

Chemicals. Commercially (Prolabo, France) available sodium arsenite $NaAsO_2$ (purity 99%), sodium arsenate $Na_2HAsO_4 \cdot 7H_2O$ (purity 98.5%), sodium dimethylarsinate (DMA) or sodium cacodylate (purity 98%) were used without further purification. The synthesis, purification and characterization of sodium methylarsonate (MMA) has been achieved in the "Service Central de Microanalyse" (Dr. A. Lamotte), CNRS, Vernaison (France).

Water used for dilutions or as buffer solution was HPLC grade (Rathburn). The phosphate buffer solution was prepared by mixing in various proportions stock solutions of 0.067 mol/l NaH_2PO_4 and 0.067 mol/l Na_2HPO_4 (Prolabo). Stock solutions of arsenical species (100 ppm) were prepared in HPLC grade water and kept at −20° C as a precaution against any degradation.

Table 1. Formulas and physical parameters of several arsenic species

Name	Chemical formula	Dissociation constant values (pKa)	UV maxima wavelengths (nm)
Arsenite		9.3	197
Arsenate		2.3 6.9 11.4	192
Monomethylarsonic acid (MMA)		2.6 8.2	192
Dimethylarsinic acid (DMA)		6.2	194
Arsenobetaine		unknown	<190
Arsenocholine		–	199

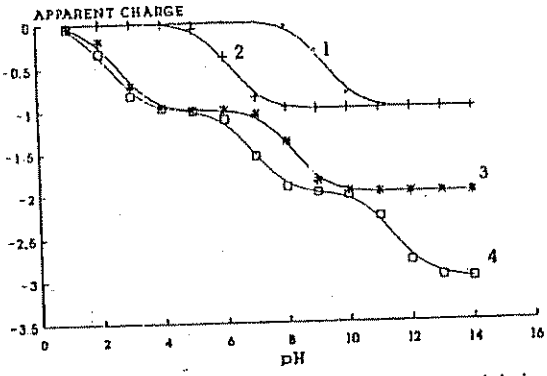


Fig. 1. Variation of the calculated charge of several arsenic ionic species versus pH. Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate

Results and discussion

Variation of the charge of arsenic species versus pH

The arsenic compounds contain acidic groups in their chemical structure, so their apparent charge depends on their pKa and on the pH of the carrier electrolyte solution (Table 1). For the arsenate anion, the apparent charge Q_{app} was calculated by the following equation:

$$Q_{app} = \frac{3(AsO_4^{3-}) + 2(HAsO_4^{2-}) + (H_2AsO_4^-)}{(AsO_4^{3-}) + (HAsO_4^{2-}) + (H_2AsO_4^-) + (H_3AsO_4)}; \quad (1)$$

where ( ) refer to the concentrations of the different forms of the trihydroxylic acid H_3AsO_4.

We have calculated the charge on each arsenic species (arsenite, arsenate, DMA and MMA) at different pH values (Fig. 1). The variation of the charge, and thus the electrophoretic mobility, versus pH, are key parameters to study the electrophoresis for these ionic species.

Effect of pH on electroosmosis

In order to determine the electrophoretic mobility of a solute by CZE, we must first consider the influence of electroendosmosis of the measurements. The apparent mobility μ_{app} is defined as:

$$\mu_{app} = L_d \cdot L_T / t \cdot V; \quad (2)$$

where L_d is the length of the capillary from the inlet to the detector, L_T the total length of the capillary, t the migration time and V the applied voltage.

In CZE, the migration time (t_0) for a neutral marker is defined as:

$$t_0 = L_d \cdot L_T / \mu_{eo} \cdot V; \quad (3)$$

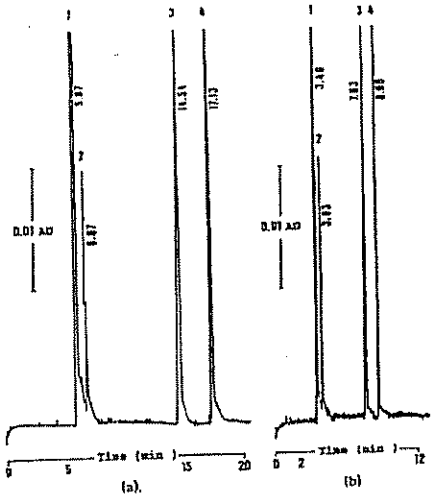


Fig. 2a, b. Effect of voltage on the separation of arsenic species. Silica capillary: 72 cm × 50 µm i.d.; carrier: phosphate buffer 25 mmol/l, pH 5.6; temperature: 30°C; detection by UV absorption at 190 nm; mixture concentration was 100 µg · ml^{-1} of each species; injected volume: 9 nl (900 pg of each species). Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate. Voltage: a 20 kV; b 30 kV

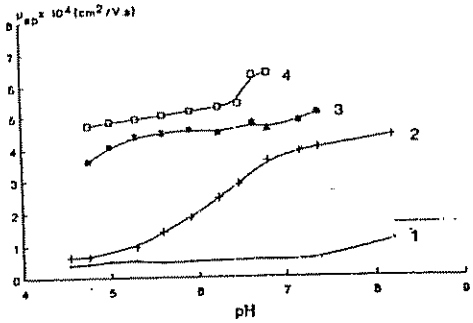


Fig. 3. Effect of pH on the electrophoretic mobilities of arsenic species. Silica capillary: 72 cm × 50 µm i.d.; carrier: phosphate buffer 25 mmol/l; applied voltage: 30 kV; temperature: 40°C. Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate

where u_{eo} is the electroendosmotic component of the apparent mobility (cm^2/V · s).

The charge of the capillary surface is greatly influenced by the buffer-pH, so differences in the electro-osmotic flow depend on pH-changes. Lambert [26] reported that the electro-osmotic mobility was dependent on the preconditioning of the fused silica column, particularly in the pH-range 4–6. The equilibration of the surface charge on the fused silica surface has been described by this author as a relatively slow phenomenon. Therefore, our capillary has been flushing between successive injections – firstly with an alkaline solution (0.1 mol/l NaOH) during 5 min – and secondly with the phosphate buffer solution during 15 min. Electro-osmotic mobility has been determined over the pH-range 4.5–8.2 in a phosphate buffer (25×10^{-3} mol/l) using methanol as neutral marker. Buffers of acidic pH reduce the electro-osmotic flow through the fused capillary, particularly in the pH range 4.5–6. For example, the electro-osmotic mobility rapidly increases from 5.6×10^{-4} cm^2/V s up to 7.9×10^{-4} cm^2/V s in the pH-range 4.5–6.2; the degree of protonation of the silanols on the capillary wall increases with decreasing buffer pH. McCormick [27] observed an interaction of phosphate with the capillary surface and mentioned that this anion strongly bound to the silica surface. The presence of these moieties on the surface of the capillary wall reduces the electro-osmotic flow by converting the residual highly acidic silanols to more easily protonated silica-phosphate complexes [27, 28].

The effect of increasing voltage in CE analysis of a mixture of these four arsenic species is shown in Fig. 2. Increasing voltage from 20 kV to 30 kV decreases the sample migration times without loss of resolution.

Effect of pH on electrophoretic mobility

When high voltage was applied, electro-osmotic flow is generated, which drives anions toward the cathode at the same velocity. Nevertheless, each anionic species is pulled back at the same time by electrophoresis depending on charge and size. The electrophoretic mobility (u_{ep}) can be expressed as:

$$u_{ep} = u_{app} - u_{eo} = L_d \cdot L_T (1/t - 1/t_0)/V \qquad (4)$$

So, the overall migration rate of these inorganic anions will strongly depend on the buffer-pH and their dissociation constants.

Some experiments were performed to further investigate the effect of pH on the selectivity and efficiency of the separation of the arsenic species. In a first experiment, the migration times were determined on consecutive injections. Table 2 summarizes electrophoretic mobilities of arsenic species calculated from the equation (1) in the pH-range 4.5–8.2. Figure 3 shows the pH-dependence of the electrophoretic mobility of As(III), DMA, MMA and As(V); neutral or alkaline pH was found to increase electrophoretic mobility depending on the dissociation constants of the species.

In a second experiment, the effect of changes of the buffer pH on the separation efficiency was investigated (Fig. 4). As(III) is completely protonated at pH 5.0 compared to the three other arsenic species whose apparent charges are −0.06 for DMA, −1.0 for MMA and −1.0 for As(V), respectively. When high voltage was applied, the electro-osmotic flow fastly drives toward the cathode the undissociated form of analyte [As(III)], and quite neutral species (DMA); anionic species, such as As(V) and MMA, moved toward the cathode with the velocity of electrophoretic migration subtracted by that of the electro-osmotic flow. For these two last anions, their weaker apparent mobilities induce longer migration times.

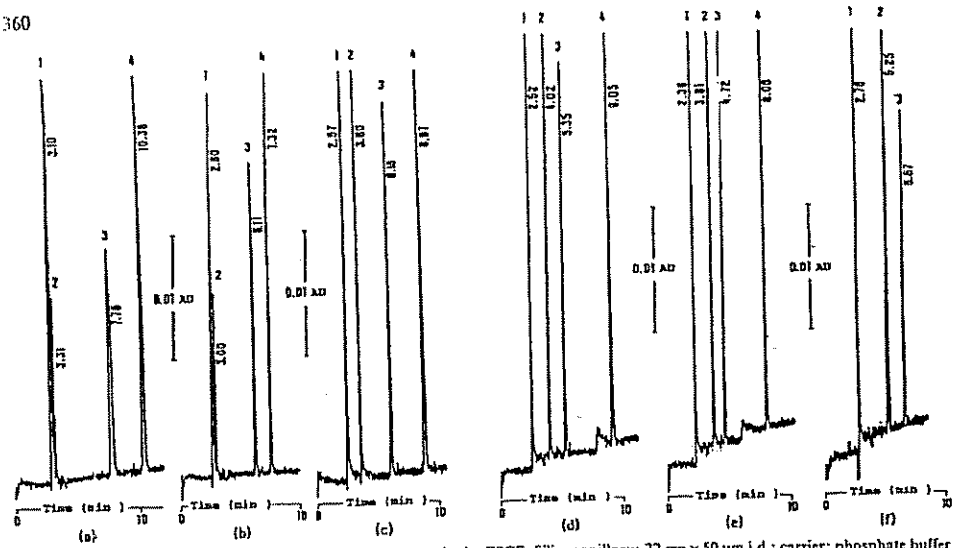


Fig. 4a–f. Effect of buffer pH on the selectivity of arsenic species by FSCE. Silica capillary: 72 cm × 50 μm i.d.; carrier: phosphate buffer 25 mmol/l; applied voltage: 30 kV; temperature: 40°C; detection by UV absorption at 190 nm; mixture concentration was 100 μg · ml^{-1} of each species; injected volume: 9 nl (900 pg of each species). Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate a pH 5.00; b pH 5.29; c pH 6.24; d pH 6.64; e pH 6.81; f pH 7.17

Table 2. Electrophoretic mobilities of arsenite, DMA, MMA, and arsenate versus pH. Silica capillary: 72 cm × 50 μm i.d.; carrier: phosphate buffer 25 mmol/l, pH 5.6; applied voltage: 30 kV; temperature: 40°C

pH	$\mu_{ep} \times 10^4$ (cm^2/V · s)			
	Arsenite	DMA	MMA	Arsenate
4.53	0.392	0.63		
4.75	0.423	0.63	3.62	4.72
5.00	0.496	0.86	4.07	4.83
5.29	0.522	0.95	4.38	4.93
5.59	0.45	1.42	4.48	5.05
5.91	0.484	1.86	4.58	5.19
6.24	0.506	2.48	4.49	5.33
6.47		2.90		5.45
6.64	0.538	3.59	4.79	6.34
6.83	0.528	3.63	4.61	6.41
7.17	0.534	3.93	4.88	
7.38	0.55	4.05	5.15	
8.20	1.15	4.40		

The selectivity α between two analytes in CZE is given by $\alpha = \delta u/u$, where δu is equal to the difference in the electrophoretic mobilities of the two analytes and u their mean electrophoretic mobility. As can be seen, the selectivity between As(III) and DMA goes from 0.47 at pH 4.75 to 1.52 at pH 7.17, an increase in selectivity of over 3 times. On the other hand, the selectivity between DMA and MMA falls from 1.30 at pH 5.0 down to 0.21 at pH 7.17, a decrease in selectivity of over 6 times. The selectivity between MMA and As(V) is equal to 0.29 in the pH-range 6.6–6.8;

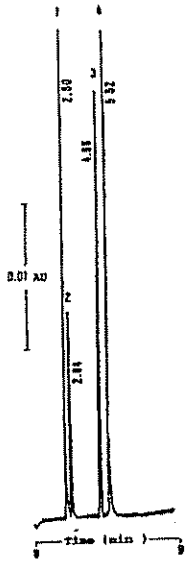


Fig. 5. Separation of arsenic species by FSCE. Silica capillary: 72 cm × 50 μm i.d.; carrier: phosphate buffer 25 mmol/l, pH 5.6; applied voltage: 30 kV; temperature: 30°C; detection by UV absorption at 190 nm; mixture concentration was 100 μg · ml^{-1} of each species; injected volume: 9 nl (900 pg of each species). Solutes: *1* arsenite (S/N = 80); *2* dimethylarsenate (S/N = 30); *3* monomethylarsonate (S/N = 50); *4* arsenate (S/N = 52)

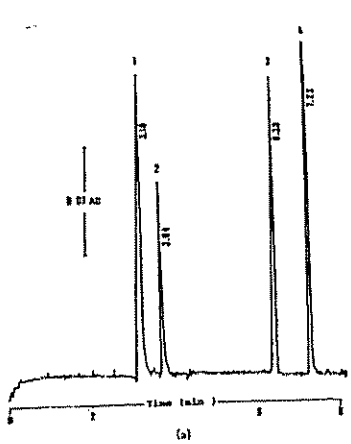


Fig. 6a, b. Effect of temperature on the separation of arsenic species Silica capillary: 72 cm × 50 μm i.d.; carrier: phosphate buffer 25 mmol/l, pH 5.6; applied voltage: 30 kV; detection by UV absorption at 190 nm; mixture concentration was 100 μg · ml^{-1} of each species; injected volume: 9 nl (900 pg of each species). Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate. Temperature: a 25°C; b 40°C

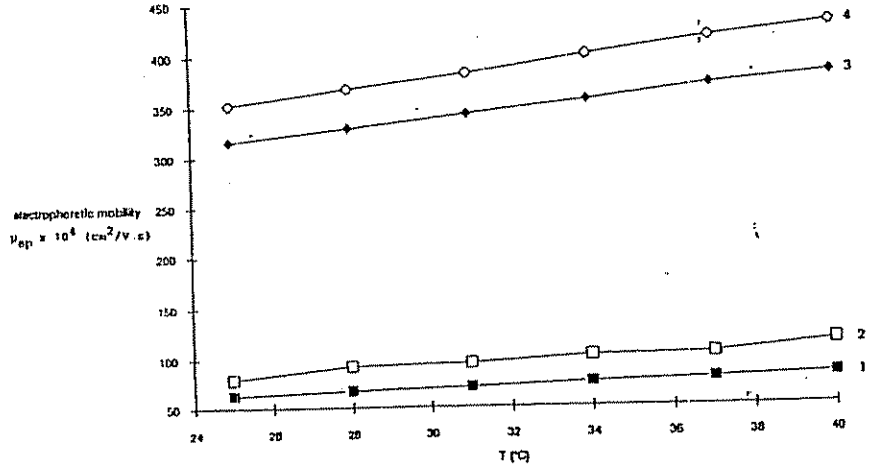


Fig. 7. Dependence of the electrophoretic mobilities of arsenic species on the temperature. Silica capillary: 72 cm × 50 μm i.d.; carrier: phosphate buffer 25 mmol/l, pH 5.6; applied voltage: 30 kV; detection by UV absorption at 190 nm; mixture concentration was 100 μg · ml^{-1} of each species; injected volume: 9 nl (900 pg of each species). Solutes: *1* arsenite; *2* dimethylarsenate; *3* monomethylarsonate; *4* arsenate

consequently, an interesting selectivity happens for a buffer pH close to 6.5 value, but the resolution always remains important in the pH range 4.5–6.8.

Arsenate and then MMA disappear from the electropherogram at higher pH-values. At pH 7.17, the velocity of electrophoretic migration for arsenate was so high that this anion (Q_{ep} = −1.65), introduced to the anodic end of the silica capillary, was forced back into the anodic buffer; consequently, only three peaks appear on the electropherogram (Fig. 4f). The same phenomenon happened for MMA above pH 8.2.

Under these conditions (phosphate buffer 25 mmol/l, pH 5.6), arsenite (t_r = 2.5 min), DMA (t_r = 2.85 min), MMA (t_r = 4.96 min), arsenate (t_r = 5.54 min) gave sharp and symmetrical peaks well resolved from each others, as shown in Fig. 5. The number of theoretical plates was rather high [132000 for As(III)], one order or more higher than the usually encountered values in HPLC. The selectivity allows

calculated charge differences of as little as 0.03 (pH 4.75) charge unit to be satisfactorily resolved.

Effect of temperature on separation

Temperature variations affect several physical parameters (viscosity, dielectric constant, dissociation constant of ionizable species, pH) and consequently the electro-osmotic flow rate and the electrophoretic mobility of the analytes. As precise control of temperature achieves more reproducible measurements of electrophoretic mobilities, temperature variations in the range 25–40°C did not significantly alter the selectivity of our separation at pH 5.6 (Fig. 6). At a temperature of 40°C, the peaks were sharper, the efficiency was slightly better and migration times were shorter. Electrophoretic mobilities are by 22%, 21%, 44% and 29% higher at 40°C than at 25°C for arsenate, MMA, DMA and arsenite, respectively (Fig. 7).

Conclusion

Free solution capillary electrophoresis is a mode of separation in CZE which mainly depends on charge difference between ionic species. In the wide pH-range (4.5–7.4), arsenite is present as the protonated (neutral) species (arsenous acid) and is swept to the detector only by the electro-osmotic flow. The other arsenic species migrate toward the cathode at different migration velocities. The selectivity and the efficiency of the separation of these four arsenic species has been improved by optimizing the buffer pH.

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Instrumentation

Influence of Temperature Control in Capillary Electrophoresis

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Key Words:
Capillary electrophoresis
Temperature control

Summary

We have investigated the influence of capillary temperature on migration time and peak area and have evaluated different cooling systems. It was found that for applied voltages below 15 kV (i.e. those most frequently used) temperature control effectively improves peak area reproducibility but has less effect on migration time.

1 Introduction

In recent years high performance capillary electrophoresis (HPCE) has been generating much interest because it provides higher separation efficiency, faster separations and lower detection limits [1, 2] than high performance liquid chromatography (HPLC).

Although the heat produced by the power consumed generating the separating potential affects the performance of CE (e.g. by decreasing resolution obtained) it has not been recognized as a serious practical problem because a narrow bore (generally 50 µm) separation tube is expected to dissipate such heat effectively owing to its large surface area to internal volume ratio. Thermal effects in HPCE can not, however, be neglected when the applied voltage becomes higher and it has been reported that without temperature control the capillary temperature can exceed 70 °C, despite the use of narrow bore tubing [3]. The effects of temperature on performance in HPCE have been reported by several researchers [4, 5] who describe, in addition to the reduction in resolution, effects on sample stability, buffer pH, buffer viscosity and other physical and chemical parameters involved in HPCE. Karger has already discussed thermal effects in terms of an Ohm's law plot of current against electric field using different cooling systems [4].

In this report, we describe the influence of the capillary temperature, employing different cooling systems, on migration time and peak area.

2 Experimental

2.1 Materials

Adenosine, AMP (adenosine monophosphate) and c-AMP were purchased from Sigma (St. Louis, MO, USA). All other reagents were from Wako Pure Chemicals (Osaka, Japan). Fused silica capillary tubing for the separations (50 µm i.d.) was obtained from Gasukuro Kogyo (Tokyo, Japan).

2.2 Apparatus

HPCE was carried out using a Jasco CE-800 system (modular type), comprising an 890-CE regulated DC high-voltage power supply, an 870-CE UV/VIS detector, and a separation unit. The power supply provides potentials up to 30 kV in 1 kV increments and is equipped with a sampling timer (0.1–999 s in 0.1 s increments) for injection by electromigration, and an analytical timer (1–999 min in 1 min increments). A current limiter is continuously setable up to 160 µA. The UV/VIS detector can be converted from HPCE to HPLC, or vice versa, simply by changing the flowcell cassette and switching the mode with a push-button selector. The separation unit comprizes electrodes with buffer reservoirs, a protective cover, and a pole for injection by the siphon effect. Injection can be performed simply by elevating the sample vial (polypropylene Eppendorf micro test tube, 1.5 ml or 0.5 ml) along the pole.

2.3 Flowcell Cassette and Capillary Separation Tube

The flowcell cassette is equipped with a condenser lens and a high gain preamplifier for maximum sensitivity. The lens is used to focus the UV radiation on to a section of the separation tube from which the protective polymer coating had been removed so that it might serve as a flowcell. The gain of the preamplifier for HPCE is ten times as high as that for HPLC. The separation tube used was a 50 µm i.d. capillary on which a 40 µm wide × 500 µm long slit had been glued (about 980 pl for a 50 µm i.d. capillary). This integrated separation tube/flowcell can be fitted to the flowcell cassette simply by placing it into a groove.

Presented at the Twelfth International Symposium on Capillary Chromatography Kobe, Japan, September 11–14, 1990.

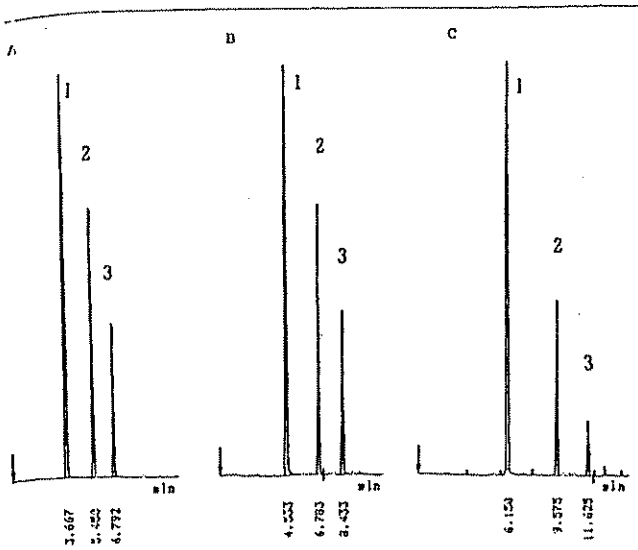

Figure 1

Electropherograms of adenines with temperature control by air circulation. Separation tube, 50 µm i.d. × 300 mm (effective length). (A) 15 °C (B) 10 °C (C) 4 °C. The electrolyte was 20 mM sodium phosphate (pH 7.0), with detection at λ = 200 nm; the applied voltage was 15 kV; currents were (A) 9 µA (B) 6 µA (C) 4 µA. The sample was (1) adenosine (2) c-AMP (3) AMP (0.03 % of each in water). Injection was by electromigration (applied voltage, 15 kV; injection time, 5 sec).

2.4 Control of Capillary Temperature

Temperature control was performed by either air circulation or water circulation (LC 101, Science Co. Ltd, Tokyo, Japan). For air circulation, the whole system was thermostatted in an environmental test chamber (Kankyo Kiki Co., Ltd, Kanagawa, Japan). In the case of water circulation, only the capillary (apart from parts of the electrodes and detection system) was thermostatted. About 55 % of the length of the capillary was temperature-controlled.

2.5 Procedure for Capillary Electrophoresis

Samples and electrolyte were contained in 1.5 ml or 0.5 ml micro test tubes which were installed in PTFE electrode blocks. A piece of platinum wire and the capillary end were positioned inside the tube in each electrode block. Injection methods employed were by electromigration and by the siphon effect. The most commonly used sampling conditions were 15 cm height for 5 s (siphon effect, about 6 nl was injected at room temperature) or 15 kV applied voltage for 5 s (electromigration). The electrolyte used for most of the experiments was 20 mM sodium phosphate at pH 7.0; a mixture of 20 mM sodium phosphate and 50 mM sodium dodecyl sulphate (SDS) at pH 7.0 was also utilized.

3 Results and Discussion

3.1 Detection Limit

L-alanyl-L-alanine (AA) and phenol in the eluate were detected with mass detection limits of 150 fmole and 51 fmole, respectively. (Injected amount, signal-to-noise ratio, S/N = 2, λ = 200 nm, at room temperature. The detection limit for peptides at λ = 200 nm was approximately half that at λ = 210 nm. The concentration and injection volume for AA were 1.5 mg/ml (dissolved in water) and about 6 nl (injected by the siphon effect), respectively. The detection limit was affected by the temperature of the capillary separation tube as well as by the ionic strength and pH of the electrolyte and sample buffer. **Figure 1** shows the electropherograms of adenines at 15 °C, 10 °C and 4 °C; as the temperature of the capillary is reduced, peak heights are lowered and migration times prolonged. For both siphon injection and injection by electromigration the injection volume decreases as the temperature is reduced.

For injection by the siphon effect, the viscosity of a sample solution increases as the temperature is reduced causing a reduction of the volume of sample introduced into the separation tube. In the case of injection by electromigration, the current decreases as the temperature is reduced owing to the reduction of ion mobility; this again results in the transfer of a smaller volume of sample. Extension of migration time is also attributed to the reduction in ion mobility.

3.2 Ohm's Law Plots

It is essential that there should be a linear relationship between current and applied voltage, i.e., Ohm's law should be valid, if reproducible results are to be obtained in HPCE: departure from a linear relationship implies that the temperature of electrolyte in the tube is being elevated by power consumption.

Results obtained using different heat removal methods, air circulation and water circulation, at 4, 10 and 25 °C were compared by investigating the dependence of current on applied voltage (*E-I* plots); the results are shown in **Figures 2** and **3**. Figure 2 shows the *E-I* plots obtained with the tube cooled by air circulation at 4, 10, and 25 °C, and that without temperature control for reference. The plots with temperature control were more linear than that without: correlation coefficients greater than 0.993 were obtained for the former case. Although there were only slight differences between plots obtained with temperature control, plots became more linear at lower temperatures. Figure 3 shows the *E-I* plots obtained with the tube cooled by water circulation at 4, 10 and 25 °C, and that without temperature control for reference. It may be observed that the efficiency of water circulation for removal of heat generated in the capillary was higher than that of air circulation. In practice, however, all the plots exhibited high linearity below an applied voltage of about 15 kV. It is, therefore, important to use a cooling system if the applied voltage exceeds 15 kV.

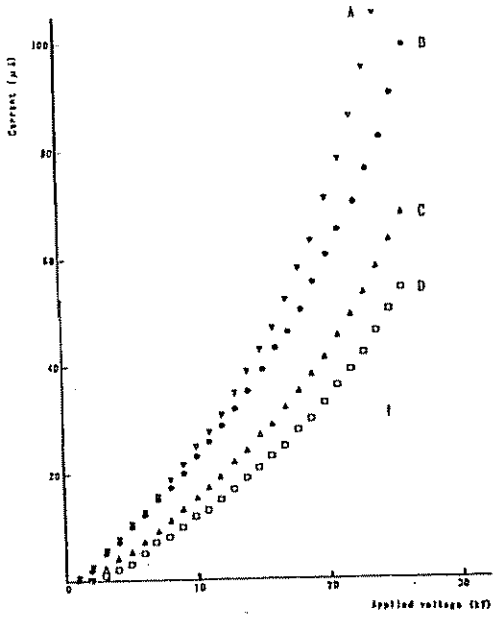

Figure 2

Ohm's law plots for temperature control by air circulation for a 50 µm I.d. × 600 mm capillary. (A) ambient (without control) (B) 25 °C (C) 10 °C (D) 4 °C. The electrolyte was that used for Figure 1. Correlation coefficients between current and applied voltage were (A) 0.975 (B) 0.983 (C) 0.985 (D) 0.986.

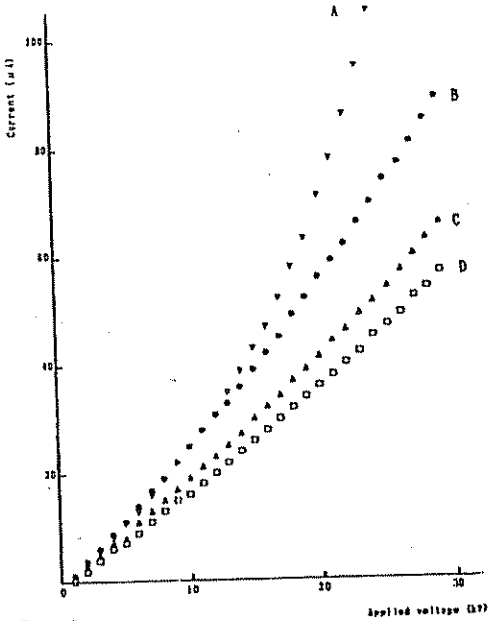

Figure 3

Ohm's law plots for temperature control by water circulation for a 50 µm I.d. × 600 mm capillary. (A) ambient (without control) (B) 25 °C (C) 10 °C (D) 4 °C. The electrolyte was that used for Figure 1. Correlation coefficients between current and applied voltage were (A) 0.975 (B) 0.998 (C) 0.999 (D) 0.999.

3.3 Reproducibilities of Migration Time (MT) and Peak Area (AR)

Table 1A shows the reproducibilities of the MT and AR of phenol under the following conditions: separation tube, 50 µm I.d. × 300 mm (effective length); electrolyte, 20 mM sodium phosphate and 50 mM SDS (pH 7.0); applied voltage, 15 kV; detection, λ = 200 nm. Temperature control was performed by air circulation. The reproducibilities of MT and AR without temperature control were 3.4 % and 9.7 %, respectively.

The reproducibility of AR when temperature control was by air circulation improved as the temperature was reduced, e.g., from 9.7 % (without control) to 2.6 % at 15 °C. Below 12 °C, the reproducibility deteriorated because the solubility of SDS decreased to such an extent that it began to precipitate from solution. When SDS was not used the reproducibility continued to increase even below 15 °C, as is shown in **Table 1B**. The improvement in the reproducibility of the migration time was however, not so significant as that of the peak area. This may be attributed to the fact that the effect of slight variations in temperature can be averaged over a long migration time.

Table 1A

Effect of capillary temperature on peak area and migration time.
Conditions: column length, 600 mm; effective length, 300 mm; I.d., 50 µm; applied voltage, 15 kV; temperature, 25 °C, 15 °C, 12 °C, and ambient (without control); electrolyte, 20 mM sodium phosphate and 50 mM SDS (pH 7.0); detection, λ = 200 nm. Injection was by electromigration (applied voltage, 15 kV; injection time, 5 s) and the sample was 656 µM phenol, dissolved in the electrolyte.

	Phenol	
Temperature	Peak area C.V.[a] [%]	Migration time C.V. [%]
Ambient	9.7	3.4
25 °C (26 µA)	6.0	4.1
15 °C (18 µA)	2.4	2.6
12 °C (17 µA)	3.5	2.7

[a] C.V. = coefficient of variation (n = 9–12).

Table 1B

Effect of capillary temperature on peak area and migration time.
Column and other conditions were as Table 1A except the electrolyte was 20 mM sodium phosphate (pH 7.0) and the sample 0.1 % AMP dissolved in water.

	AMP	
Temperature	Peak area C.V.[a] [%]	Migration time C.V. [%]
25 °C (13 µA)	6.8	0.9
15 °C (9 µA)	6.6	0.7
10 °C (6 µA)	3.5	1.4
4 °C (4 µA)	3.8	1.2

[a] C.V. = coefficient of variation (n = 8–10).

No averaging effect can be expected for injection volume, however, as injection takes place in a very short time, e.g. 5 s. Because of this a slight change in temperature may considerably effect the injection volume, leading to poor reproducibility

As is shown in **Table 2** the reproducibilities of MT and AR when temperature control was performed by water circulation were significantly better than those obtained using air circulation. It is considered that thermal contact between water and the capillary tube is so good that the heat generated in the tube is effectively removed.

In conclusion, for applied voltages below 15 kV, such as are most frequently used, temperature control effectively affords better peak area reproducibility but is not so effective with migration time. If better peak area and migration time results are required when applied voltages higher than 15 kV are being used, the temperature should be maintained below room temperature by water circulation

Table 2

Effect of cooling technique on reproducibility.
Conditions: applied voltage, 15 kV at 10 °C ; other conditions as in Table 1A.

	AMP			
Temp.	Air control		Water control	
	Peak area C.V. [%]	Migration time C.V. [%]	Peak area C.V. [%]	Migration time C.V. [%]
10 °C	3.6	1.4	2.0	0.6

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UNITED STATES PATENT AND TRADEMARK OFFICE

Application of

Applicant : Lenore Kelly, Dean S. Burgi, Robert J. Nelson
Serial No. : 08/088,439
Filing Date : July 7, 1993
Title : CONTROLLED TEMPERATURE ANION SEPARATION BY CAPILLARY ELECTROPHORESIS
Docket : SPA 096 PA
Art Unit : 1102

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231, on October 11, 1993.

Attorney 29,001 Reg. No.

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and Trademarks
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This Information Disclosure Statement is filed within the period set forth in §1.97(b) because it is believed to be filed before the mailing date of a first Office Action on the merits.

A listing of all items of information cited is attached on PTO Form 1449. Copies of the items are enclosed for the convenience of the Examiner.

Respectfully submitted,
KILLWORTH, GOTTMAN, HAGAN & SCHAEFF

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ATTY. DOCKET NO.: SPA 0416 PA | SERIAL NO.: 08/038,439

APPLICANT: Kelly et al

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U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	5,066,383	11/19/91	Weinberger et al	204	299R	
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	AC	5,128,005	7/7/92	Jones et al	204	180.1	
	AD	5,156,724	10/20/92	Jones et al	204	180.1	
	AE	5,167,791	12/1/92	Glatz	210	656	
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	AR	Small et al, "Indirect Photometric Chromatography," American Chem. Soc. 1982
	AS	Foret et al, "Indirect Photometric Detection in Capillary Zone Electrophoresis," Elsevier Science Publishers B.V. 1989
	AT	Kelly et al, "Separation of Small Anions using Dichromate for Indirect UV Detection," Research Disclosure, August 1992

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OTHER PRIOR ART (Including Author, Title, Date, Pertinent Pages, Etc.)

	AR	L. Kelly, "Separation of Organic Acids Using Phthalate Ion for Indirect UV Detection" publ. in Research Disclosure Aug. 10, 1993
	AS	L. Kelly et al, "Capillary Zone Electrophoresis of Organic Acids and Anions" J. of Liq. Chrom. Vol. 16 Nos. 9&10 pp. 2103-2123 1993
	AT	

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single yellow color of the solution as a precision dose unambiguously indicating the partial conversion to the four-valent state, is clearly visible even after only ½ h of equilibrium time. After 24 h equilibration the reduction is almost complete and the distribution coefficients obtained are very similar to those for vanadium(IV).

Coefficients for chromium(III) depend very markedly on the history of the solution. Very indistinct bands representing various chromium ion species appear in column operations at room temperature. This is due to the slow ligand exchange rates of chromium(III).

Although the potential for element separation in the tartrate system is high more attractive alternatives are usually available. A molecule such as tartrate often is troublesome to remove. Only when it is already present in the system or has been added to prevent hydrolysis of elements such as tantalum and niobium should separations in tartrate media be considered as a first choice. The outstanding exception to this philosophy is the group separation of Zr, Sn(IV), etc. from all other elements that was considered earlier in the discussion. The only alternative would mean the use of a system containing hydrofluoric acid.

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Indirect Photometric Chromatography

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Indirect photometric chromatography is a sensitive single-column ion analysis method developed from the concept that photometers may be used to detect transparent ionic species. The use of light-absorbing eluent ions in an ion-exchange mode enables sample ions to appear as "troughs" in the base line absorbance as transparent sample ions substitute for the light-absorbing displacing ions. The elution times of these troughs vary with the ion injected and their depths (or areas) are proportional to the amount of sample injected. Notable advantages of the new technique are its single column simplicity, its applicability to a wide range of ionic species, and an inherently greater sensitivity than single-column conductometric approaches.

Ion determination by liquid chromatography is often frustrated not by separation problems but by detection problems. An example is the problem of determining the many important inorganic ions that are not light-absorbing. Whereas the separation of such transparent ions may be conveniently effected on ion exchange resin columns, their detection and measurement by conventional photometric means are thwarted since they are optically indistinguishable from the transparent eluents commonly prescribed.

The technique known as ion chromatography (*1*, *2*) was developed to circumvent the detection problem posed by transparent sample ions. In just 6 years it has become a widely practiced and popular method addressing problems in a great variety of areas (*3*). Ion chromatography (IC) usually comprises a two-column arrangement followed by a conductance detector where the first column serves to separate the ions of interest while the second column, the suppressor, serves to lower the conductance of the eluent while usually increasing the conductance of the eluted sample ions. The suppressor column in IC becomes exhausted in the course of normal usage and must be periodically regenerated or replaced—usually regenerated. Whereas this regeneration step has been automated in commercial instruments so that it is relatively unobtrusive or is made continuous as in the recently developed hollow fiber suppressor (*3*), it would nevertheless be desirable and advantageous for the following reasons to develop single column (suppressorless) methods for the many non-chromophoric ions.

(1) Decreased complexity of the instrumentation should yield a concomitant increase in reliability. This is a very important factor in penetrating the process control area with chromatographic methods where the demands for unattended and relatively maintenance-free operation have high priority.

(2) Reduced dead volume as a result of eliminating the suppressor will yield faster analysis and somewhat improved resolution.

Suppressorless single-column conductometric methods of ion analysis have been described in earlier literature (*4*–*7*). The limitations of these approaches have been elaborated by Pohl and Johnson (*8*) who point to the problem inherent in attempting to determine accurately the often times small changes in eluent conductivity that accompany the replacement of eluent ions by sample ions—both of which are conducting. They argue the sensitivity advantage to be gained by adding a suppressor that effectively "removes" the conductance of the eluent.

We would like to report a single column approach that solves the monitoring problem in a different manner while retaining much of the sensitivity of the original ion chromatography method. This new technique is derived from a comprehensive development of the concept that photometers may be used to monitor the many "transparent" ionic species

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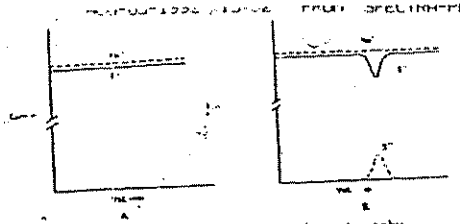


Figure 1. Principle of indirect photometric chromatography.

commonly thought not to be amenable to this type of detection. Although mentioned earlier by Leurent and Bourdon [9], lack of sensitivity was expected to limit application of the new idea. The independent discovery and development of the method as reported here demonstrate its abundant versatility, particularly in optimizing sensitivity.

From the standpoint of sensitivity we will also discuss how this novel means of detection overcomes the problems that are intrinsic to suppressorless conductometric monitoring.

A feature of this new photometric approach is the use of light-absorbing (usually UV absorbing) eluents, made so by including in the eluent light-absorbing ions of the same charge as the ions to be separated.

These light-absorbing ions have a dual role: (1) of selectively displacing the sample ions from the chromatographic column; (2) of revealing the sample ions in the effluent.

The appearance of sample ions in the effluent is signaled by "dips" or "troughs" in the base line absorbance of the effluent as the transparent sample ions substitute for the light-absorbing displacing ions.

In applying this new approach to the very sensitive detection and determination of ions, it is essential to understand the interplay of such factors as concentration of eluent, concentration of sample, capacity of the ion exchanger, and the optical properties of the eluent. The definition of these critical relationships occupies a large part of this contribution.

We have chosen the name indirect photometric chromatography (IPC) to describe this rapid, sensitive, broad scope exploitation of ion exchange and photometric monitoring.

PRINCIPLE OF THE METHOD

Consider an ion exchange column—for illustrative purposes specifically an anion exchanger—which has been pumped and equilibrated with an electrolyte denoted Na^+E^- so that the sites in the exchanger are occupied exclusively by eluent ions E^-. A concentration monitor capable of sensing all ionic species and placed at the outlet of such an operating column would reveal a steady level of Na^+ and E^- if the feed concentration of the eluent is maintained constant (Figure 1A). If an injection is made of sample electrolyte, denoted Na^+S^-, then the sample anion, S^-, will generally be retarded by the stationary phase and will exit at a characteristic elution volume determined by such factors as the capacity of the exchanger, the concentration of the solution, and the affinity of the stationary phase for S^- relative to E^-. A suitable monitor at the column exit would indicate the concentration of S^- to rise and fall in a familiar fashion as it leaves the column (Figure 1B). Conventional ion exchange LC art has been concentrated on devising suitable detectors for *directly* monitoring the magnitude (height or area) of these sample peaks. Generally ignored has been the fact that accompanying the appearance of S^- there must be a concerted and equivalent change in E^- since, by the principles of electroneutrality and equivalence of exchange, the total equivalent concentration of anions (S^- and E^-) must remain fixed since the concentration of sodium cations is fixed. It therefore follows that the concentration of S^- in the effluent could be *indirectly* monitored by continuously monitoring the level of eluent ion E^-.

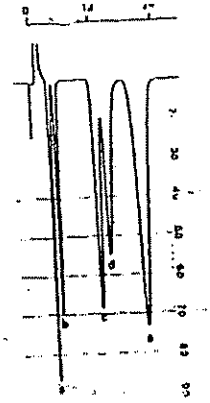


Figure 2. Separation and indirect photometric detection of several "transparent" sample ions: (a) chloride, (b) nitrite, (c) bromide, (d) nitrate, (e) sulfate.

On the basis of this argument it follows how this somewhat latent feature of the ion exchange mode may be usefully tapped in the case of problematical sample ions. Thus, if sample ions are inconveniently lacking in a particular property, for example, optical absorbance, one may exploit this deficiency in the sample species by deliberately choosing an eluent ion that *is* light absorbing and monitoring the "troughs" generated in the base line absorbance as transparent sample ions elute.

The development of this combination of ion exchange and indirect photometric monitoring is the main concern of this contribution. An example may serve to illustrate how the method works:

A column containing an anion exchanger was equilibrated with a dilute (10^{-3} M) solution of [redacted] until the effluent absorbance was stable as indicated by the UV photometer monitoring the column effluent. When a sample containing chloride, nitrite, bromide, nitrate, and sulfate was injected, the chromatogram of Figure 2 was obtained. By making separate injections of the individual anions in the mixture, we established the identities of the troughs in phthalate absorption. The off-scale positive deflection is due to the ion exchange displacement of phthalate by the injected sample anions as a whole. Since the total equivalent concentration of the sample exceeded that of the eluent, the void disturbance was positive. When the total concentration of the sample is less than that of the eluent, the disturbance is negative.

An elution order was established experimentally for a large variety of anions using phthalate as displacing ion. This is recorded in Table I which may be used as a rough guide in predicting the feasibility of certain separations. But it must be used judiciously for, as will be evident later, elution order depends upon a variety of experimental conditions. The feasibility of determining ions by this indirect approach brings a number of questions to mind, notably, how does one choose an eluent species from the enormous number that qualify through being ionic and having appropriate spectral properties? What are appropriate spectral properties? What is the sensitivity of the method and how is it related to the various elution conditions? These questions were addressed in a

Table I. Elution Volume of [illegible] ions[a]

ion	V_E, mL	ion	V_E, mL
void	2.0	fluoride	3.0
acetate	3.2	glycolate	3.4
azide	5.5	iodate	3.8
bromate	14	malesate	7.8
bromide	6.9	malonate	6.2
carbonate	6.5	nitrate	8.4
chlorate	8.8	nitrite	24.1
chloride	17.1	o-phosphate	7.5
monochloracetate	8.0	propionate	3.3
dichloracetate	27.5	succinate	4.7
trichloracetate	b	sulfate	11.4
citrate	b	sulfite	8.6
cyanate	6.5		

[a] Eluent: 10^{-3} M sodium phthalate, 10^{-3} M boric acid, pH 9. Column: 4 × 250 mm, SAR-20 0-6. [b] Very large.

systematic manner—the results and recommendations follow.

CONSIDERATIONS IN THE CHOICE OF ELUTING IONS AND CONDITIONS

In IPC, sample ions are revealed and quantified by the decrements they produce in eluent concentration. Since the displacing species is usually in much greater abundance than sample species—a feature of elution chromatography—these decrements would ordinarily represent rather small fractional changes in eluent level. Thus, the successful application of IPC is directly related to how precisely we can measure these fractional differences (the signal) in the presence of the random fluctuations (the noise) of the base line response. To this end it is critical to the understanding of IPC to appreciate how signal to noise ratio and, in turn, sensitivity are related to an important variable in the system, namely, the concentration of the eluent.

(A) Concentration of the Eluent. Let us consider the case of elution of a sample ion through an anion exchanger operating in the IPC mode. It is assumed that the conditions have been chosen so that the eluted sample is adequately remote from the void disturbance. Typically this would result in a Gaussian-shaped change in sample ion concentration, but for simplicity of treatment we will assume that it emerges as a square wave pulse with maximum concentration, C_S. This pulse of sample will cause a concomitant and identically shaped pulse change in the eluent level as indicated in Figure 3A. The signal to be measured, ΔS, is the difference between the signals due to the eluent at base line concentration, C_E, and when sample elutes, $C_E - C_S$.

This may be expressed as follows:

$$\text{SIGNAL} = \Delta S \propto C_S A_S + (C_E - C_S)A_E - C_E A_E = C_S(A_S - A_E) \quad (1)$$

where A_S and A_E denote the absorptivities of the sample and eluent ions, respectively. Equation 1 assumes that the signal response is directly proportional to species concentration.

Figure 3A depicts an ideal, that is, a noiseless detection situation. Reality is represented in Figure 3B which depicts the noise within which the signal must be detected, that is, the uncertainty in measuring the concentration of eluent C_E. At a given base line absorbance, noise represents a fixed (random) fluctuation, represented by N. The signal as a fraction of the base line absorbance is given by the expression

$$\frac{C_S(A_S - A_E)}{C_E A_E} \quad (2)$$

from which it follows that

$$\frac{\text{signal}}{\text{noise}} \propto \frac{C_S(A_S - A_E)}{NC_E A_E} \quad (3)$$

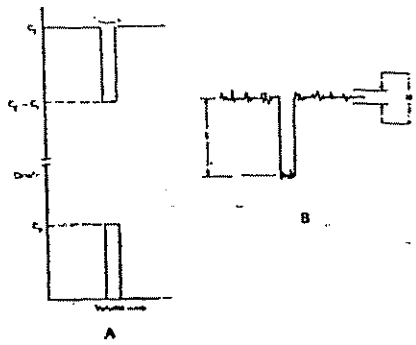


Figure 3.

For transparent ions A_S is zero so, neglecting signs which are not significant for our purposes

$$\frac{\text{signal}}{\text{noise}} \propto \frac{C_S}{NC_E} \quad (4)$$

This simple expression incorporates the conclusion that sensitivity improves, the lower the concentration of eluent employed.

There are other important considerations, however, that impose lower limits to the concentration of eluent preferred. Perhaps most significant among them are in the penalties of overlong run times and loss of sensitivity due to band spreading that result from using an eluent that is too dilute. Ideally, the run time should be no longer than the time necessary to adequately resolve the troughs.

When using LC in an ion exchange mode, there is a further very fundamental consideration to be kept in mind when choosing the concentration of the eluent, that is, the saturation level in the eluting capacity of the eluent. How close one operates to this level, or to what extent one exceeds it, exercises strong control over the sharpness of the eluted peaks and hence resolution. Basically the source of the limitation derives from the inability of eluent to displace sample ion at a higher concentration than that of the eluent.

(B) The Relative Affinity of E^- and S^-. As well as its concentration, the displacing power of the eluent ion with respect to the sample ions is an extremely important factor in the practice of IPC. Different ions vary widely in their displacing power. In an attempt to reduce these eluent options to a reasonable number, we examined a large variety of candidate eluents having a wide range of ion exchange affinities and from these limited our recommendations, somewhat arbitrarily, to just a few. [redacted] Trimesate and sulfobenzoate are generally more potent displacing ions than phthalate while iodide is less so. This is illustrated in Figure 4 which is a plot of the elution volume of sulfate ion on a surface agglomerated anion exchanger for the four different eluent species as a function of eluent concentration. Within the concentration range of eluent depicted, the eluting power of the displacing ion is seen to follow the expected trend of polyvalent ions being more potent displacing species than monovalent ions. This order of potency is not always obeyed for, as will be seen shortly, it depends on the charge on the eluent and sample ions and on the concentration of the eluent.

(C) The Effect of the Charge of E^- and S^- on Elution Rate and Elution Order. The elutability of several sample

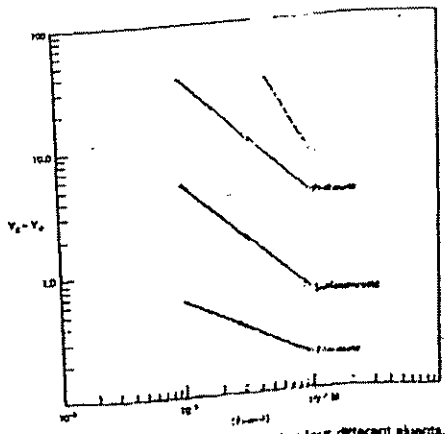


Figure 4. Elution volumes of sulfate ion using four different eluents. Resin: 2.8 × 250 mm, SAR-40-0.8

ions by the four candidate eluents was measured, and the results are illustrated in Figure 5. The quantity $V_R - V_e$ is the corrected elution volume of the ion, that is, the effluent volume between sample injection and through elution less the void volume of the column. A number of features of the data are noteworthy: (1) the linear dependence of log $(V_R - V_e)$ on log (concentration of eluent); (2) the differing slopes of the log $(V_R - V_e)$ vs. log C plots (There are in fact five different slopes in the 15 plots of Figure 5); (3) the cross-over in certain elution orders.

The elution can thus be described in the convenient mathematical form

$$\log (V_R - V_e) = -m \log [E^{n-}]$$

where $[E^{n-}]$ is the molarity of the eluent.

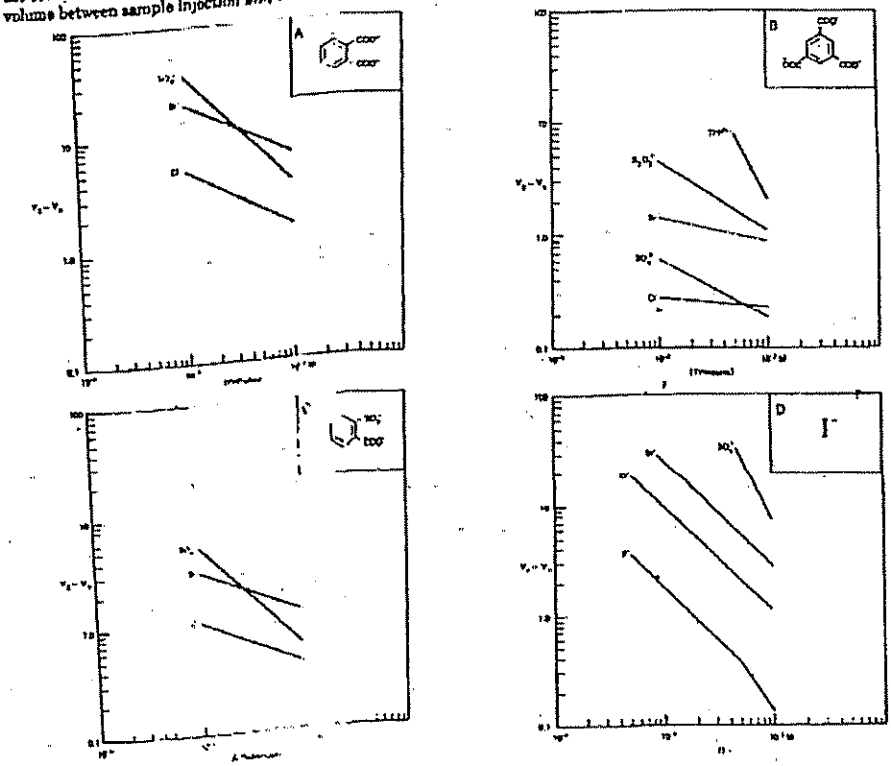
A relationship of this form, namely

$$\log (V_R - V_e) = \text{constant} - y/x \log [E^{n-}] \qquad (5)$$

may be developed from basic ion exchange theory (10).

This expression, besides accounting for the experimentally observed linearity in the log $(V_R - V_e)$ vs. log [eluent] plots, also defines the slope as the ratio y/x of the charge of the sample ion to that of the eluent ion. Consequently, it is of interest to examine how the experimentally observed slopes agree with the values predicted by the expression. Accordingly, the slopes of the plots in Figure 5 were measured and are compared with the values predicted by eq 5. The comparison is provided in Table II and with one exception—trimesate eluent, bromide sample ion—the agreement between theoretical and observed is good to excellent.

This has an important practical implication. It suggests that from a knowledge of the charges of the sample and eluent



[illegible] (A) phthalate; (B) trimesate; (C) sulfobenzoate; (D) iodide. Resin: 2.8 × 250 mm, SAR-40-0.8.

Table II. Comparison of y/x (Theoretical) with y/x (Observed)

E^{z-}	S^{y-}	y/x	y/x obsd
I^-	Br^-	1	1.00
I^-	SO_4^{2-}	2	2.00
trimesate^{3-}	Br^-	1/3	0.21
trimesate^{3-}	SO_4^{2-}, $S_2O_3^{2-}$	2/3	0.60
trimesate^{3-}	TPP^{7-}	?	1.9
phthalate^{2-}	Br^-	1/2	0.47
	SO_4^{2-}	1	0.98

ions and a single determination of V_R at a single concentration of eluant, it is possible to predict V_R for that particular species at other eluent concentrations. Furthermore, the dependence of slope on ion charge ratio explains the observed cross-overs in elution order. Knowing the origin of this effect in turn affords us another means of controlling resolution and avoiding the condition of nonresolvability that is represented by a cross-over point.

PHOTOMETRIC FACTORS

The precise determination of eluent absorbance (concentration) is a very important part of IPC, so principles that apply to conventional spectrophotometric measurements also apply to IPC. It is known from classical spectrophotometry that the most accurate measurements are obtained when the optical density is 0.43 (*11*). Actually this value is not critical and the error of measurement varies little within a range of optical density from about 0.2 to 0.8. For this reason it is important to the accuracy of IPC to monitor the eluent under conditions where its absorbance falls within this range.

Since the concentration of eluent to be used will generally be dictated by such other considerations as column capacity and eluent ion affinity and since cell path length is fixed, by what means may optical absorbance be adjusted? The molar absorptivity of a given eluent ion generally exhibits so great a dependence upon wavelength, that the desired eluent absorbance is obtained merely via selection of detector wavelength. A photometric detector with multiwavelength monitoring capabilities is therefore a very useful adjunct to IPC although under certain conditions fixed wavelength devices have operated quite effectively. Eluent concentrations as dilute as 10^{-6} M and as concentrated as 1 M have conformed to the "optimum absorbance" requirement of IPC through appropriate choice of detection wavelength.

APPLICATIONS

Experimental Section. The apparatus used in IPC is conventional, usually consisting of an eluent reservoir, a pump, a sample injection valve, an ion exchange column, a flow-through photometric detector, and a recorder. A trough integrator is optional.

Any of a large variety of LC pumps is suitable—in this work the Laboratory Data Control (LDC) Constametric I, the LDC minipump, and the Altex Model 110A were used. Photometers found to be useful were two fixed-wavelength types, the LDC Model 1203 and the Altex 153, and two multiwavelength instruments, the Perkin-Elmer LC-75 and the Varian UV-50. Note that the photometric detector responds to small fractional reductions in an elevated base line as solute ion zones traverse the optical cell. This feature calls for techniques to suppress the base line level without reducing or otherwise obscuring the small troughs. We have achieved this in two ways. In dual beam instruments with reference cells we have operated with the reference cell containing either air or eluent. Operating with air in the reference cell we found that some of the commercially available photometers did not have enough zero adjustment to null out the high absorbance of some eluents, and we found it necessary, therefore, to develop additional base suppression circuitry to handle this problem. Operation with eluent in the reference cell avoids the problem.

In a few cases we found careful temperature control of the column to be necessary. In one eluent system—the trimesate at pH 10—we found an exceptional sensitivity to temperature change in the column. For example, it would manifest itself in marked biphasic base line disturbances on even slight warming of a short section of column as might be brought about by briefly gripping the column between finger and thumb. Enclosing the column in a thermostating bath maintained at 37 ± 0.1 °C eliminated this problem. As a rule, however, we found such close temperature control to be unnecessary.

The eluent in IPC is only slightly changed on passing through the column and may be recycled to the eluent reservoir with negligible penalty. This ability to recycle eluent is desirable in unattended process control applications.

Stainless steel columns (4.1 × 250 mm) or glass columns (2.6 × 250 mm) were used to contain the ion exchangers, and sample was introduced via a Rheodyne Model 7010 injector.

The column packings are one of the most important features of IPC and a variety of materials are effective. We have made wide use of ion exchangers originally developed for ion chromatography (*1, 12–14*). Especially useful are the surface agglomerated pellicular anion exchangers which are prepared by depositing a monoparticulate layer of submicron anion exchanging spheres onto a much larger (20–50 μm) substrate bead whose surface is anionic. In the original IC work (*1*), surface sulfonated styrene divinylbenzene copolymer particles were much used as substrate spheres but in the present research, conventional strong acid cation exchangers such as Dowex 50 have been used exclusively for this purpose. The very small particle anion exchangers of uniform particle size distribution were prepared by quaternizing emulsion copolymers of vinylbenzyl chloride and divinylbenzene (*14*). Surface agglomeration was carried out by adding a quantity of the substrate cation exchange resin to a suspension of the colloidal anion exchanger. The anion exchange capacity of these surface agglomerated separating resins is controlled by the size of the colloidal particles and by the size of the substrate, being greater the smaller the substrate size and decreasing as the colloidal resin size decreases. Surface agglomerated resins are described in the text by a code that indicates the sizes of the substrate and of the colloidal anion exchanger. For example, the designation SAR-20-0.6 denotes a surface agglomerated resin prepared by coating a 20 μm diameter cation exchanger with a 0.6 μm diameter colloidal anion exchanger.

Ion exchangers found to be useful in IC for cation analysis are also useful in IPC applications. In this regard, surface sulfonated styrene divinylbenzene copolymer spheres (*12*) have been used to develop a number of IPC analysis schemes. Besides these low capacity ion exchangers, high specific capacity materials have been successfully applied to IPC. They are especially useful in applications where direct injection of concentrated samples is desirable and column overloading becomes an important consideration.

A number of commercial anion and cation exchangers have been used with success.

The following applications have been chosen to exemplify the scope, selectivity, and sensitivity of IPC. Details on columns, packings, eluents, etc. are provide in Table III.

Anion Separations. Figure 6 represents the separation of a mixture of five anions. Noteworthy is the trough produced by carbonate ion. This ability to determine anions of high pK acids gives IPC an advantage over ion chromatography which by its nature is very insensitive to such species.